Cytokine and acute phase protein mRNA expression in liver tissue from pigs with severe sepsis caused by intravenous inoculation of Staphylococcus aureus

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ABSTRACTS

1

IL-6 Mediates Elevation Of Acute-Phase Proteins But Does Not Affect Adipose Tissue Inflammation In Obesity Induced By High-Fat Feeding In Mice

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Obesity is associated with adipose tissue (AT) inflammation, increased levels of acute-phase proteins and chronically elevated levels of IL-6. To assess the role of IL-6 in modulating obesity-induced AT inflammation and hepatic acute-phase response, we induced obesity by high-fat diet (HFD) in WT and IL-6 KO mice. Feeding a HFD for 13 weeks induced a comparable degree of obesity, hyperleptinemia, hyperglycemia and hyperinsulinemia in WT and KO mice compared to their respective chow-fed lean controls. A significant increase in mRNA expression of adhesion molecules (VCAM-1 and ICAM-1), markers of macrophage infiltration (CD68 and CD206), cytokines and chemokines (TNF alpha, IL-1Ra, CXCL2, CCL2, CCL11) was observed in obese compared with lean mice irrespective of genotype. No significant differences between obese WT and KO mice were observed for any of the parameters evaluated. In contrast to lack of effects on AT inflammation, IL-6 deficiency was associated with a significant reduction in hepatic mRNA expression and circulating protein levels of serum amyloid A (SAA), a major acute-phase reactant. These results indicate that the chronic increase in IL-6 levels associated with obesity does not significantly modulate AT inflammation but is responsible for mediating induction of the hepatic acute-phase response.

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2

Immunosenescent Macrophages Cannot Control Streptococcus Pneumoniae Colonization, Resulting In Invasive Pneumococcal Disease

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Over 90% of pneumonia deaths occur in the elderly, the majority due to *Streptococcus pneumoniae*. Although age is a risk factor for many infectious diseases, the exceptionally high burden of *S. pneumoniae* infection that occurs despite vaccination, implies that there are *S. pneumoniae* specific defects in host defence. Colonization of the nasopharynx, which is generally asymptomatic and transient, is an essential pre-requisite to infection and is controlled in large part by resident and recruited macrophages. We have developed a mouse model of *S. pneumoniae* nasopharangyeal colonization using clinical isolates with varying invasive capacity and have compared innate immune responses between young (8-10wk) and aged mice (14mo+). Aged mice have higher bacterial loads in the nasopharynx and develop fulminant pneumonia when colonized with *S. pneumoniae* strains that are non-invasive in young mice indicating that they are unable to contain colonization in the nasopharynx. We have recent unpublished data that suggests that recognition and containment of *S. pneumoniae* colonization by macrophages is mediated by non-opsonic receptors, a process that appears to be impaired in macrophages from aged mice. Interestingly recognition by non-opsonic receptors is essential for downstream immune events such as cytokine production and antibody production and may surpass the importance of the Toll like receptors during the aging process. In order to confirm the importance of these processes in humans we will monitor the kinetics of *S. pneumoniae* colonization in a cohort of nursing home patients.

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3

The Role Of ICAM-3 In The Removal Of Apoptotic Cells

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Damaged, aged or unwanted cells are removed from the body by an active process known as apoptosis. This highly orchestrated programme results in cell disassembly and the exposure of 'flags' at the dying cell surface that permit recognition and removal by viable cells (phagocytes). Efficient phagocytic removal of dying cells is essential to prevent inflammatory and autoimmune disorders.

Relatively little is known of the molecular mechanisms underlying changes at the apoptotic cell surface. We have previously shown that ICAM-3 (a heavily glycosylated, leukocyte-restricted Immunoglobulin Super-Family member) undergoes a change of function as cells die so that it acts as a molecular 'flag' to mediate corpse removal. Our work seeks to characterise apoptosis-associated changes in ICAM-3 and define their role in ICAM-3's novel function in apoptotic cell clearance.

Here we extend earlier studies to show that apoptotic cell-associated ICAM-3 functions, at least minimally, to tether apoptotic leukocytes to macrophages via an undefined receptor. Whilst CD14 has been suggested as a possible innate immune receptor for apoptotic cell-associated ICAM-3, we demonstrate ICAM-3 functions for apoptotic cell clearance in the absence of CD14. Our data additionally indicate, that during apoptosis, leukocytes display early changes in cell surface glycosylation and a marked reduction in ICAM-3, a change that correlates with a reduction in cell volume. This reduction in ICAM-3 is explained by cell surface shedding of microparticles ('apoptotic bodies') that contain ICAM-3. Such microparticles, released from apoptotic leukocytes, are strongly chemoattractive for macrophages. In addition, microparticles from ICAM-3-deficient leukocytes are significantly less chemoattractive than microparticles from their ICAM-3-replete counterparts.

Taken together these data support the hypothesis that ICAM-3 acts as an apoptotic cell-associated ligand to tether dying cells to phagocytes in a CD14-independent manner. Furthermore our data suggest that released ICAM-3 may promote the recruitment of phagocytes to sites of apoptosis.

4

Impaired Bacterial Clearance In Cutaneous Wounds From Aged Mice Is Associated With Decreased Neutrophil Accumulation

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Advanced age is associated with an impaired innate immune response which increases susceptibility to infection, contributing to delayed dermal wound closure and elevated rates of wound dehiscence in the elderly population. To further determine the role of innate immune dysfunction in the age-associated inadequate response to wound infection, young (10-12 weeks) and aged (18-20 months) BALB/c mice were subjected to 3mm dorsal punch wound before receiving *Staphylococcus aureus* (100-125 CFU/wound). At 24 hours after injury and infection, bacterial colonization within the wound was elevated 45-fold ($p<0.05$) in aged mice compared to young mice. By 72 hours, the bacterial burden at the injury site was significantly increased ($p<0.0003$) in aged mice ($6.63x10^5$ CFU/mL) in contrast to young mice ($1.36x10^5$ CFU/mL). At 24 hours after wound alone or wound and infection, the percentage of neutrophils at the injury site remained constant in young mice (~20%). In contrast, aged mice demonstrated a 42% decrease in neutrophil accumulation in wounded tissue following injury and infection as compared to dermal injury alone ($p<0.04$). Moreover, in response to cutaneous injury and infection, neutrophil CXCR2 expression was reduced ~19% in aged mice

($p<0.03$) as compared to young. Together, these data suggest aberrant neutrophil function as a contributor to reduced bacterial clearance which may subsequently impair dermal wound closure. This work is supported by the NIH R01AG018859 (EJK), T32AA013527 (EJK), F31AA017027 (JK), R01AA015731 (MAC), Ralph and Marian C. Falk Research Trust and the MD/PhD Program at Stritch School of Medicine.

5

Innate and Adaptive Immune Responses in the Pathogenesis of Septic Shock

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Septic shock is widely recognized as a state of systemic immune dysregulation and coagulopathy produced by invasive microorganisms and/or their microbial products. What is less clear is the most appropriate intervention to re-establish control over these dysregulated host inflammatory networks once the process has begun. Early therapeutic strategies to eliminate the microbial infection, re-establish effective blood flow and prevent or treat organ dysfunction are the traditional management approaches for septic shock. Direct interventions targeting the early events in innate immune activation during sepsis have not yet been successful clinically. A large phase III international trial with a specific MD2-TLR4 inhibitor has just been completed and the results of this clinical trial should be available soon. Major problems with this approach have been the short time interval available to intervene effectively and the multitude of interacting inflammatory networks that are activated simultaneously in sepsis. An alternative strategy now gaining favor is directed at the counter anti-inflammatory response to sepsis. Many septic patients rapidly develop significant immunosuppression and a number of novel agents are now available to attempt to re-establish immune competence. The treatment of clinical septic shock will not be easy and will likely necessitate a personalized medicine approach to optimally manage the complexity of sepsis.

6

The Innate and Adaptive Immune Response to Shock/Trauma

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Severe traumatic injuries and burns elicit nearly uniform activation of innate immune system modules. Although the proximal inciting mechanism(s) has not been clearly identified, much evidence points to alarmin molecular pattern recognition as an essential signal. Several early and persistently expressed transcriptional themes in immune cells point to Toll-like receptor 4 (TLR4) activation as a principal pathway for early innate immune activation. In addition to molecular pattern/alarmin signals, the immune response to traumatic challenge may also be modified by other interacting systems, including efferent neuro-endocrine influences. The innate immune response may also be shaped by pre-existing organ specific and systemic host allostatic factors. The traditional view of acquired “immune paralysis” resulting sterile injury implies an altered Th1/Th2 ratio. The innate response may also be modulated by subsets of regulatory T-cells (Treg).These interactions are better defined in animal models of injury than among injured humans. In addition, other Treg populations (Th17) may modify both innate and adaptive responses. The confounding influences of the clinical care environment will be discussed in the context of this existing knowledge of the innate and adaptive immune interface.

7

An Innate Immune-Enhancing Whole Cell Therapeutic For The Treatment Of Acinetobacter Baumannii Pneumonia

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Acinetobacter baumannii is a significant cause of nosocomial infections worldwide. The growing burden of *A. baumannii* disease together with alarming rates of antibiotic resistance in this organism have galvanized efforts aimed at developing novel therapeutics to treat *A. baumannii* infections. We set out to identify therapeutic targets founded upon our previous work linking NaCl exposure to increased colistin tolerance in *A. baumannii*. Toward this end, we generated a transposon mutant library and screened for mutants that fail to demonstrate NaCl-induced colistin resistance. This screen identified over 30 candidate genes including a gene encoding a predicted glycosyltransferase (LpsB) involved in synthesis of the lipopolysaccharide core. As expected, disruption of *lpsB* results in increased sensitivity to colistin and loss of NaCl-induced colistin resistance. In addition, Δ*lpsB* exhibits a greater than 6-log reduction in virulence in a murine model of *A. baumannii* pneumonia, irrespective of antibiotic treatment. Strikingly, co-infection with wildtype and Δ*lpsB* leads to significant attenuation of wildtype *A. baumannii*. Inflammatory gene expression profiling and measurement of immune cell recruitment suggest that the therapeutic effect of Δ*lpsB* stems from differential regulation of key inflammatory molecules and increased neutrophil recruitment to the lungs. These findings suggest that Δ*lpsB* may be an effective therapeutic tool to harness the host inflammatory response and direct it toward bacterial clearance. Accordingly, we evaluated the therapeutic efficacy of chemically killed Δ*lpsB* for the treatment of *A. baumannii* pneumonia. Treatment with killed Δ*lpsB* cures mice of Acinetobacter pneumonia by 36 hours post infection. Taken together, these data demonstrate that LpsB is a viable therapeutic target, and establish Δ*lpsB* itself as a novel whole cell therapeutic for *A. baumannii* pneumonia. Moreover, our data suggest that Δ*lpsB* exerts its therapeutic effect by enhancing the host inflammatory response to promote bacterial clearance. Future work will capitalize on these findings by optimizing the therapeutic potential of Δ*lpsB* and employing this strain as a tool to define the components of an effective host response against *A. baumannii*.

8

Triggering CLEC5A On Immature Myeloid Cells Results In Enos-Dependent Lethal Shock

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C-type lectin domain family 5, member A (CLEC5A), aka myeloid DAP12 associating lectin-1 (MDL-1), is a myeloid receptor that signals through DAP12 and activates multiple downstream kinases. Although no known endogenous ligand of CLEC5A has yet been identified, dengue virus has been shown to bind and activate CLEC5A on macrophages, resulting in inflammatory cytokine and chemokine production. We observed that mice treated with concanavalin A (ConA) underwent a massive G-CSF-dependent hepatic infiltration of $CLEC5A^+$ leukocytes. Subsequent activation of CLEC5A with an agonist antibody (DX163) resulted in lethal shock within 30 minutes that was associated with elevated levels of serum nitric oxide (NO) and TNF-α. We employed both chemical inhibitors and knockout mice to delineate the signaling pathways involved in CLEC5A-mediated shock. Our results showed that: (i) surprisingly, the induction of shock required eNOS, but not iNOS, to modulate TNF-α production via regulation of TACE activity; (ii) both DAP10 and DAP12 signaling adaptor molecules, as well as Syk and PI3K were important for CLEC5A-mediated lethal shock. CLEC5A expressing cells isolated from livers of ConA-treated mice are $CD11b^+$/Gr-

1^+/$Ly6G^+$/$Ly6C^+$/$eNOS^+$ with ring-shaped nuclei, consistent with immature myeloid cells. *In vitro* activation of CLEC5A on these myeloid cells resulted in production of nitric oxide and TNF- α. Furthermore, *in vivo* depletion of Gr-1^+ or $Ly6G^+$ cells conferred complete protection against shock, indicating that these immature myeloid cells are pathogenic in this model. Finally, we showed that triggering CLEC5A using inactivated dengue virus rather than DX163 also resulted in NO and TNF- α production *in vitro* as well as lethal shock *in vivo*. Our work suggests that pathologic NO and TNF- α production from immature myeloid cells may play a role in dengue shock syndrome as well as other systemic inflammatory responses. Inhibition of the CLEC5A-Syk-PI3K-eNOS-TACE pathway may be of therapeutic value in these settings.

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9

Leishmania Exosomes Modulate the Properties of Human Monocytes and Dendritic Cells to Confer Immunosuprressive Phenotypes

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We recently reported that leishmania use exosomes as a general mechanism for protein secretion and that these vesicles are involved in the delivery of proteins to host target cells (Silverman et al., *J. Cell Sci.* 2010). In the present study, we investigated the properties of leishmania exosomes with respect to influencing innate and adaptive immune responses mediated by primary human myeloid cells. E xosomes from *Leishmania donovani* modulated monocyte cytokine responses to γ-interferon in a bimodal fashion by promoting IL-10 production and inhibiting that of TNF- α. Moreover, these vesicles were inhibitory with respect to both MHC class II expression on the surface of CD40L matured monocyte-derived dendritic cells (MoDCs) as well as cytokine responses (IL-12p70, TNF- α , IL-10). Consistent with this immunosuppressive phenotype, exosomes from *Leishmania major* promoted Th2 polarization in infected BalB/c mice and exacerbated infection with *L. major*. Exosomes from wild-type *L. donovani* were also ineffective at priming MoDCs to drive the differentiation of naïve CD4 cells into γ-interferon producing Th1 cells. In contrast, vesicles from HSP100 null *L. donovani* showed a gain-of-function phenotype that was pro-inflammatory and promoted the differentiation of naïve CD4 lymphocytes into Th1 cells. Proteomic analysis also showed that exosomes from wild-type and HSP100 null *L. donovani* had distinct differences in protein cargo, suggesting that packaging of proteins into leishmania exosomes is regulated in part by HSP100. Furthermore, mutant exosomes, unlike WT vesicles, promoted CD4 and CD8 T-cell proliferation as well as pro-inflammatory cytokine production in *L. donovani* infected C57BL/6 mice in an antigen specific manner. T hese findings demonstrate that l eishmania exosomes are capable of immune-modulation and that they are predominantly immunosuppressive. To our knowledge, this is the first evidence to suggest that changes in the luminal protein cargo of exosomes may influence the impact of these vesicles on myeloid cell function.

10

Alternative TLR Signaling Restores Francisella tularensis LPS-mediated Protection against Francisella tularenis LVS Challenge in TLR2-/- Mice

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The Category A select agent *Francisella tularensis* (*Ft*) is the causative agent of tularemia, a potentially fatal infectious disease. Intraperitoneal (i.p.) infection of mice with ~10 *Ft* Live Vaccine Strain (*Ft* LVS) organisms causes a lethal infection that resembles human tularemia. Previous studies have revealed that immunization with *Ft* LVS lipopolysaccharide (*Ft*-LPS) just 2 days before bacterial challenge provokes a B1a cell-derived antibody response that protects wild-type (WT) mice against an otherwise lethal *Ft* LVS challenge. Herein we show that this same regimen offers limited protection to $TLR2^{-/-}$mice (WT, 85% survival *vs*. $TLR2^{-/-}$, 25% survival, $p < 0.0001$, N = 4 separate experiments) and IRAK4 Kinase Dead Knock-In ($IRAK4^{KD-KI}$)mice (WT, 100% survival *vs*. $IRAK4^{KD-KI}$, 18 % survival, p = 0.0004, N = 3 separate experiments). *Ft*-LPS exhibits no TLR2 agonist activity, however macrophage recognition of *Ft* LVS is overwhelmingly TLR2-dependent (Cole, et al. Infect. Immun. **2007** 75 (8) 4127-37), and IRAK4 is a pivotal protein kinase in the MyD88-dependent signal transduction pathway. Therefore we hypothesized that treatment of $TLR2^{-/-}$ mice with an alternative, MyD88-dependent, TLR agonist would compensate for reduced recognition of *Ft* LVS in $TLR2^{-/-}$ mice, thereby enhancing *Ft*-LPS-mediated protection. Administration of the TLR4 agonists, protein-free *E. coli* K235 LPS or synthetic *E. coli* monophosphoryl lipid A (MPL), a non-toxic derivative of *E. coli* lipid A, at the time of either *Ft*-LPS immunization or *Ft* LVS challenge, increased the survival of *Ft*-LPS-pretreated $TLR2^{-/-}$ mice to 100%, while treatment of mice with MPL, in the absence of *Ft*-LPS, conferred partial protection against *Ft* LVS challenge for both WT (76% survival) and $TLR2^{-/-}$ (55% survival) animals. This suggests that effective *Ft*-LPS-mediated protection against *Ft* LVS challenge requires two discrete recognition signals, *i.e*., *Ft*-LPS recognition and MyD88-dependent, TLR signaling.

11

Role Of Lipid A Modification In H. Pylori-Host Interactions

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Modification of bacterial surface structures, such as the lipid A portion of lipopolysaccharide (LPS), is used by many pathogenic bacteria to help evade the host innate immune response. *Helicobacter pylori*, the only gram-negative bacterium capable of chronic colonization of the human stomach, modifies its lipid A by removal of phosphates from the 1 and 4' positions of the lipid A backbone. In this study, we identify the enzyme responsible for dephosphorylation of the lipid A 4̣-phosphate in *H. pylori* ($LpxF_{HP}$). To ascertain the role these modifications play in the pathogenesis of *H. pylori*, we created mutants in *lpxE* (1-phosphatase), *lpxF* (4̣-phosphatase) and a double *lpxE/F* mutant. Mass spectrometry analysis of the single *lpxE* and *lpxF* mutants revealed lipid A with a 1 or 4̣-phosphate respectively, while the double *lpxE/F* mutant revealed a *bis*-phosphate lipid A species. Mutants lacking *lpxE, lpxF, or lpxE/F* show a 16, 360, and 920-fold increase in sensitivity to the antimicrobial peptide (AMP) Polymyxin B (PMB), respectively. Moreover, a similar loss of resistance is seen against a variety of AMPs found in the human body including LL37, β-defensin 2, and P-113. Interestingly, by using a fluorescent PMB derivative in a novel binding assay, we show that unlike wild-type, the *lpxE/F* mutant's membrane is able to bind PMB, presumably due to changes in the net charge of lipid A. The LPS of *lpxE*, *lpxF* and *lpxE/F* mutants show a 2.5, 12.5, and 24-fold increase in Toll-like Receptor 4 activation, respectively. Furthermore, a mouse colonization study revealed greatly reduced colonization of the *lpxE/F* mutant when compared to wild type. Our results demonstrate that modification of lipid A in *H. pylori* is key to its ability to colonize and cause disease. Development of inhibitors that target these modification enzymes could lead to effective antimicrobial treatments.

12

Temperature Regulated N-Acyltransferases (Lpxd) Contribute To Lipid A Structural

Heterogeneity And Pathogenesis In Francislla Tularensis Subspecies Novicida.

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Francisella tularensis, an intracellular Gram-negative pathogen causes the disease tularemia in animals and humans. Lipopolysaccharide (LPS), a major component of the bacterial outer membrane plays an essential role in maintaining membrane integrity and is a key factor in host innate immune recognition. The lipid A component of LPS from Francisella subspecies is different from those of classical enteric bacteria in terms of its structure and biological activities. Interestingly, Francisella synthesize lipid A with an altered distribution of fatty acids in response to growth at environmental (21ºC) versus host (37ºC) temperatures. Mass spectrometric analysis clearly shows that this heterogeneity is due to alterations mainly at the N-linked hydroxy fatty acids present on the diglucosamine backbone of lipid A. In this study, we indentified two N-acyltransferase enzymes, designed as LpxD1 and LpxD2 present in the genomes of all Francisella subspecies. These two enzymes share 34% amino acids homology.

LpxD1 and LpxD2 deletion mutant strains were made by allelic exchange. Complete lipid A structural analysis showed that the LpxD1 enzyme preferentially added a 18-carbon hydroxy fatty acid at 37ºC, whereas the LpxD2 enzyme added a 16-carbon hydroxy fatty acid at 21ºC. Heterologous expression of the individual enzymes in *E. coli* confirm that these acyltransferases added long chain fatty acids to the lipid A backbone. Transcriptional analysis using microarray and real-time PCR confirmed that the individual acyltransferase genes were regulated similarly as observed for the structural analysis (eg *lpxD1* is up regulated at 37ºC and *lpxD2* is up regulated at 21ºC). Finally, to evaluate the potential role of these enzymes in pathogenesis, we tested both deletion mutants in mice using a subcutaneous route of infection. LpxD1-null mutants were attenuated in mice and can protect mice from lethal wild-type challenge. This stain may be useful for immunization against tularemia.

13

Promoting Resolution of Inflammation: A Novel Genus of Pro-Resolving Chemical Mediators

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Mechanisms in timely resolution of the acute response can give new insights in host defense. Using a systems approach with self-limited inflammatory exudates to map tissue events, cell traffic and identification of protein and chemical mediators, we identified new families of potent bioactive lipid-derived mediators, coined resolvins and protectins in resolving exudates using lipid mediators lipidomics. Each of these pro-resolving mediators controls the duration and magnitude of acute inflammation in vivo with stereospecific sites of action in the pico- to nanogram range. The mapping of these endogenous resolution circuits provides new avenues to harness uncontrolled inflammation and consider the molecular basis of many inflammation-associated diseases (C Serhan et al

Nature Reviews Immunology 2008). This SLB/IEIIS presentation will overview on our recent advances on the biosynthesis and functions of this novel genus of specialized pro-resolving mediators (SPM). These structurally distinct families of local chemical mediators were originally identified in murine exudates captured during the natural self-limited resolution phase and are also produced by human isolated cells. SPM include 3 families of chemical mediators resolvins, protectins and the most recent addition, maresins. These are biosynthesized from essential omega-3 fatty acids (EPA and DHA) and possess potent multi-pronged anti-inflammatory, pro-resolving and anti-microbial actions in murine models of sepsis (Spite et al Nature vol. 461, 2009). The actions of SPM proved to be potent, cell type-specific and stereoselective with isolated human cells and in animal diseases. We also used microfluidics to monitor single cell actions of SPM in tandem to LC-MS-MS lipidomics within 1 μl^3 volume < 5 min from whole blood. Endogenous formation of resolvins and protectins and their organ-protective roles were confirmed by others and extended into clinical trial. For example, SPM have potent actions and are formed in murine ischemic renal injury (Hassan and Gronert JI 2009) in obesity-induced insulin resistance and liver disease (Gonzalez-Periz et al FASEB J 2009) and in reducing pain (Xu et al, Nature Med. 2010) . Identification of endogenous SPM biosynthesized during acute inflammation-resolution indicates that the resolution of acute inflammation is an active programmed process. These findings change the >200 year old concept that *resolution of inflammation is a passive process*. Taken together they indicate that natural resolution pathways may underlie many prevalent diseases associated with uncontrolled inflammation and open the potential for resolution-based pharmacology. The author acknowledges support of NIH grants DE019938, NS067686, GM038765 and DK07448.

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14

Adminstration of TAT-Hsp27 protein exacerbates immune complex-mediated lung injury in rats by promoting neutrophil survival

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Scaffolding protein Heat shock protein 27 (Hsp27) positively regulates Akt signal complex. Disruption of Akt-Hsp27 interaction by transduction of TAT-Akt-117-128 peptides or anti-Hsp27 antibody but not TAT-scrambled peptides or isotype control antibody prevents Akt activation and induces neutrophil apoptosis. To directly examine role of Hsp27 in regulating neutrophil survival during inflammation, we determined effects of transducing TAT-control and TAT-Hsp27 proteins in rats subjected to immune complex-mediated acute lung injury.

Appropriate TAT fusion-proteins were administered intratracheally, 2 hr after initiation of lung injury. After additional 2 hr, we collected bronchoalveolar lavage (BAL) fluids and separated BAL cells and BAL supernatants. We determined vascular permeability index after injury in the presence and absence of each TAT protein. Lung injury caused a significant increase in vascular permeability index which was further increased by administration of TAT-Hsp27 as compared to TAT-vector control. Concomitant with increased neutrophil recruitment, H&E staining of lung tissue sections demonstrated that TAT-Hsp27 administration exacerbated edema and hemorrhage in the lung tissue as compared to control animals treated with or without injury. TAT-Hsp27 transduction after initiation of lung injury enhanced neutrophil survival as demonstrated by increased Akt phosphorylation in BAL PMNs compared to TAT-control treated lung injured animals. Increased neutrophil survival induced caspase-3 activation in lung tissue from lung injured animals treated with TAT-Hsp27 but not TAT-control proteins. In addition, we demonstrated enhanced release of C-reactive Protein (CRP) in BAL supernatants obtained from injured animals treated with TAT-Hsp27 compared to injured animals treated with TAT-control protein. Furthermore, PAI expression was enhanced in injured animals treated with TAT-Hsp27 compared to TAT-control protein. These results demonstrate an important role for Hsp27 in regulating Akt activation and neutrophil apoptosis. Thus, Akt117-128 peptides can be used as therapy to treat imflammatory diseases by promoting neutrophil apoptosis to ameliorate neutrophil-mediated organ injury.

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NALPs and Innate Immune Recognition of Infectious Agents

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A rapidly emerging field is the study of a large family of mammalian genes encoding proteins with a conserved nucleotide-binding domains (NBD) and leucine-rich repeats (LRR). This family has been referred to as the NLR family. This family of genes is found in all mammalian species examined, and even preserved in sea urchins.

In this talk, the role of NLR proteins in regulating host response to microbial pathogens will be discussed. The range of microbes that is sensed by NLRs include bacteria, virus, fungi and parasites. A common mechanism is likely to be involved. In addition recent evidence indicates a strong role for these proteins in the regulation of inflammation, cancer and autoimmunity through changes in the adaptive immune response.

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Resolvin E1 Regulates Activation of Human Platelets in Response to ADP

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Cardiovascular diseases are the leading cause of death in the Western World. Results from several clinical studies indicate that diets rich in marine fish omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic (EPA) can reduce the risk of coronary events. Resolvin E1 (RvE1) is a potent EPA-derived specialized pro-resolving mediator biosynthesized during resolution of acute inflammation. RvE1 exhibits organ-protective actions in vivo and acts on specific cell types such as platelets. Here, we investigated the ability of RvE1 to regulate

adenosine diphosphate (ADP) activation of platelets mediated via specific receptors because RvE1 reduces ADP-stimulated platelet aggregation. RvE1 (0.1-100nM) incubated with platelets gave reduced ADP-stimulated P-selectin mobilization (IC_{50} ~$1.6x10^{-12}$ M) and polymerized actin compared to control platelets. RvE1 (1-100 nM) did not stimulate or block intracellular calcium mobilization. Using a new $P2Y_{12}$-β-arrestin-coupled cell system, ADP-activated $P2Y_{12}$ with an EC_{50} of $5x10^{-6}$ M and RvE1 (0.01 nM- 1 uM) did not directly stimulate $P2Y_{12}$ or block ADP-$P2Y_{12}$ signals. Another mediator in this system, LTE_4 (EC_{50} $1.3x10^{-11}$ M) dose dependently activated $P2Y_{12}$. When recombinant $P2Y_{12}$-expressing cells were transiently transfected with an RvE1 receptor, human ChemR23 (present on human platelets), addition of RvE1 (0.1-10nM) blocked ADP signals (IC_{50} ~$1.6x10^{-11}$) in $P2Y_{12}$-ChemR23-expressing cells compared to mock transfections. These results demonstrate that RvE1's regulatory actions (i.e reducing ADP-stimulated P-selectin mobilization and actin polymerization) are hChemR23-dependent. Moreover, these findings can provide novel approaches for platelet therapies as well as explain, in part, the actions of dietary EPA via local RvE1 production and platelet interactions that impact cardiovascular diseases and a timely resolution of acute inflammation. (Supported by NIH grant nos. GM38765, R01DE019938-01 and RC2AT005909).

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Pro-inflammatory neuropeptides that signal via neurokinin-1 receptor promote potent type-1 biased immunity

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There is evidence supporting that the nervous system regulates the outcome of innate and adaptive immunity. Accordingly, the tachykinins substance-P and hemokinin-1 are proinflammatory neuropeptides that favor cellular immunity, by binding agonistically the neurokinin 1 receptor (NK1R). Dendritic cells (DCs) known to link innate and adaptive immunity, express functional NK1R. However, the ability and mechanisms employed by NK1R agonists to modulate the immune-stimulatory and -biasing functions of DCs are unknown. Here, we analyzed the effects of NK1R-signaling of murine bone marrow derived myeloid DCs (BMDCs) on their abilities to initiate and bias T-cell immunity. We demonstrate that BMDCs express functional NK1R and the expression of the receptor increased significantly following exposure to LPS as demonstrated by qRT-PCR. Agonistic signaling via the NK1R promoted the maturation and diminished significantly the secretion of IL-10 by BMDCs stimulated or not with LPS, without increasing secretion of IL-12p70. Adoptive transfer of NK1R-signaled BMDCs to C57BL/6 mice induced potent Ag specific CD4+ (Th1) and CD8+ (CTL/Tc1) immunity. By comparing the responses elicited by NK1R-signaled BMDCs in C57BL/6-wild type, -IL-12p35-/- and -CD11c+DTR mice (the latter conditionally depleted of endogenous conventional DCs) we demonstrated that the development of the type-1 immunity, required lacking of IL-10 by the adoptively transferred BMDCs and secretion of IL-12p70 by endogenous DCs. In conclusion we demonstrated that inflammatory signaling of myeloid DCs via NK1R results in potent Th1 and Tc1 immunity mediated by pro-inflammatory DCs with ability to affect the immune stimulatory function of endogenous DCs.

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Regulation Of The Immunological Activity Of Lipopolysaccharide By Surfactant Protein A

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Endotoxin (Lipopolysaccharide, LPS) is a strong stimulus of the human immune system. The lung is constantly exposed to endotoxin from inhaled microorganisms. Therefore a tight regulation of the immune response in order to balance constant inflammation versus pathogen defense is required. The pulmonary surfactant protein A (SP-A) is one of the primary secreted components of lung innate immunity mediating essential anti-inflammatory functions. SP-A has been shown to bind to LPS, however, the mechanisms underlying SP-A-mediated immune modulation are only partially understood.

The presence of SP-A instantly attenuated the proinflammatory immune response of HEK293-TLR4/MD2 cells to LPS without the need of a preincubation suggesting a direct effect of SP-A on LPS. Physicochemical properties of LPS have been shown to critically determine its biological activity. Therefore, we analyzed the molecular consequences of SP-A interacting with LPS in a series of experiments on SP-A/LPS complexes. We determined the molecular conformation and the three-dimensional aggregate structure of aequeous LPS preparations in the presence of SP-A by small-angle-X-ray-scattering (SAXS). These experiments showed, that SP-A induces structural changes in LPS aggregates leading to a biologically less active conformation of LPS. Determination of the IR-absorbance of the 1- and the 4'- phosphate groups of LPS showed, that SP-A induced a shift of the absorbance of the hydrated 1-phosphate, suggesting a specific interaction of the protein with this phosphate. To investigate the biological activity of SP-A/LPS complex formation we measured TNF α -cytokine expression in human macrophages. These experiments confirmed that the SP-A/LPS-interaction reduces the biological activity in human macrophages.

In conclusion, our experiments demonstrate that LPS/SP-A-interaction induces conformational changes in LPS-aggregates leading to biologically less active structures, thereby providing a new mechanism of immune modulation by SP-A.

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CD93 deficiency increases susceptibility to peritonitis

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CD93 is a transmembrane glycoprotein expressed on myeloid cells, endothelial cells, stem cells, plasma cells, and platelets. CD93 regulates several processes critical to inflammation including phagocytosis and adhesion; however, the molecular function of CD93 and its role in disease has been elusive. Recent *in vivo* studies in mice, and genome wide studies in humans, support a role for CD93 in the regulation of inflammation. We previously reported that soluble CD93 (sCD93), produced by inflammatory macrophages, is elevated in mouse peritoneal lavage fluid following thioglycollate (TG) induced peritonitis and endotoxin induced sepsis. CD93-deficient mice have increased leukocyte infiltration 24 hours post injection of TG, which returns to WT levels by 96 hours. Here we extend the kinetics and demonstrate a 1.5-1.7 fold increase in the number of leukocytes in the peritoneal cavity of $CD93^{-/-}$ mice compared to WT mice at 3, 6 and 12 hours post injection of TG. sCD93 levels were highest in peritoneal fluid at 3 hours post injection of TG correlating with increases in proinflammatory cytokine production. Conversely, CD93-deficient and WT mice were

equally susceptible to endotoxin induced sepsis, and as expected no difference in the number of leukocytes in the peritoneal cavity was observed at 6 or 18 hours post intraperitoneal injection of LPS. Moreover, there was no detectable difference between WT and CD93$^{-/-}$ mice in proinflammatory cytokine/chemokine levels in peritoneal lavage fluid as assessed by Luminex multiplex cytokine array and ELISA. These measurements included CCL21, which has been suggested to be a mediator of elevated leukocyte recruitment in the CD93$^{-/-}$ mice. TG induced peritonitis resulted in greater leukocyte recruitment into the peritoneal cavity compared to endotoxin induced inflammation, whereas proinflammatory cytokine levels were more upregulated in septic mice. Taken together these data indicate that CD93/sCD93 regulates leukocyte infiltration, however CD93's role in proinflammatory cytokine/chemokine production remains unclear.

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Micro-RNAs Expressed by Subsets of Myeloid-derived Suppressor Cells

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Myeloid derived suppressor cells (MDSC) are components of the suppressive immune system that can be increased by either infection or tumor growth. Murine MDSC can be divided into monocytic-MDSC (Mo-MDSC) or neutrophlic-MDSC (PMN-MDSC) based on surface expression of Ly6C or Ly6G respectively. However, a marker defining human MDSC's remains to be identified because humans lack the genes for the Ly6G/C surface markers. Further CD11b+Ly6G+ or Ly6C+ cells can be purified from naïve animals, but they lack suppressive activity. Thus better markers and/or more knowledge of the molecular basis for MDSC function is needed in order to define the human MDSC subset and target them for therapeutic intervention. Therefore, we isolated CD11b+Ly6C+ or CD11b+Ly6G+ splenocytes from naive Balb/c or mice bearing 4T1, and performed microRNA array analysis comparing the naïve CD11b+Ly6C+, CD11b+Ly6G+ to tumor-bearing CD11b+Ly6C+ and CD11b+Ly6G+ cells. From the arrays we identified 6 differentially expressed microRNAs. One microRNA, miR-155, was elevated in both tumor subsets, which was anticipated. Three microRNAs, namely miR141*, miR340-5p and mmu-miR-883a-5p, were confirmed. Since mmu-miR-883a-5p is only expressed in mice, we have focused on miR141* and miR340-5p, which are expressed in many species including human. MiR340-5p is highly expressed by murine tumor PMN-MDSC and also highly expressed by the human cell line HL60. MiR141* is highly expressed by tumor MDSC and has previously been shown to be highly expressed by solid tumors, where it is thought to regulate migration of tumor cells through both SMAD and PTEN pathways. These data lead to the hypothesis that miR141* and mir340-5p are select markers of functional MDSC.

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Resolvin D1 specific binding with neutrophils and monocytes and identification of pro-resolving receptors

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Pro-resolving mechanisms in acute inflammation are essential for host defense and the return to homeostasis. Resolvin D1 (RvD1; 7*S* , 8*R* , 17*S* -trihydroxy-4*Z* , 9*E* , 11*E* , 13*Z* , 15*E* , and 19*Z* -docosahexaenoic acid) is a newly discovered

pro-resolving and anti-inflammatory mediator biosynthesized from docosahexaenoic acid (DHA) via lipoxygenases during resolution. RvD1 displays potent, stereoselective actions that include limiting further neutrophil infiltration and stimulating macrophage phagocytosis of apoptotic cells as well as microbial particles. In the present experiments, RvD1 decreased actin polymerization and blocked leukotriene B_4-regulated β2 integrins on human neutrophils in a pertussis toxin sensitive fashion. We prepared a synthetic [^{3}H]-RvD1 and found specific RvD1 recognition sites on human neutrophils with a Kd ~0.2 nM. Receptor screening systems were used to identify RvD1 receptors. Two candidates were identified, ALX, a lipoxin A_4 receptor, and human GPR32, an orphan to date—that were further validated using a β-arrestin-based ligand receptor system. In addition, RvD1 enhanced macrophage phagocytosis of both zymosan and apoptotic neutrophils which was increased with overexpression of human ALX and GPR32. RvD1-enhanced phagocytosis was decreased with selective knockdown of these G-protein coupled receptors. Structure-function relationship for RvD1 was examined using the β-arrestin system. Human GPR32 was also activated by aspirin-triggered RvD1 and 17(R/S)-methyl-RvD1, a metabolically stable analog of RvD1 with similar potencies. In sharp contrast, neither DHA nor 17-oxo-RvD1, an inactive further metabolite of RvD1 activated GPR32. Taken together, these results indicate that RvD1 specifically interacts with both ALX and orphan GPR32 on human neutrophils and mononuclear cells, and RvD1-receptor interactions are stereoselective. This study was supported in part by the National Institutes of Health Grants GM38765 (to C.N.S.), and 1RC2AT005909 (to C.N.S.). [SK and NC contributed equally]

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Partially oxidized phospholipids of microvesicles represent an endogenous danger signal activating TLR4

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OBJECTIVES: TLR4 plays a role in the pathophysiology of numerous diseases without the presence of bacterial LPS. The main question is what is the common pathophysiological denominator of all those disease conditions? Numerous proposed TLR4 agonists in fact function as chaperones of LPS. The presence of oxidative stress and reactive oxygen species has been shown in all of those diseases, but the molecular mediator between ROS and activation of TLR4 as well as the molecular mechanism of receptor activation has been missing. RESULTS: Under the oxidative stress human cells, such as platelets or endothelial cells release lipid microvesicles (MVs). We found that MVs isolated from the plasma of patients with rheumatoid arthritis induce activation of Toll-like receptor 4 (TLR4)/MD-2 complex but not other TLRs, leading to MyD88 and TRIF-dependent cytokine production and MAPK signalling. Inactive MVs from healthy donors and synthetic MVs can be converted to TLR4/MD-2 agonists by limited oxidation, while prolonged oxidation abrogated the activity, identifying partially oxidized phospholipids as the active constituent. MD-2 binds anionic phospholipids and TLR4 activation is inhibited by LPS antagonists. Molecular docking suggests that receptor dimerization is mediated by polar oxidized acyl chain protruding from the hydrophobic binding pocket of MD-2 to interact with TLR4 ectodomain, triggering receptor activation based on the similar interactions as activation by LPS.

CONCLUSIONS: Partially oxidized phospholipids of MVs represent an early endogenous danger signal and elucidate the pervasive role of TLR4 signalling in inflammation.

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Microvesicles are a Common Means of Macrophage Activation by Pathogens and Tumor

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Immunomodulatory microvesicles are produced by a wide variety of cells, including tumor cells. These microvesicles are important for shaping the local immune response. Recently, we found that microvesiculation can also be induced by treatment of cells with sublytic doses of the bacterial toxin streptolysin O. In order to determine how these microvesicles can activate macrophages, we examined bone-marrow derived macrophages treated with microvesicles by immunohistochemistry and FACS. We find that these microvesicles are endocytosed by macrophages, which upregulate CD69, and costimulatory molecules CD80 and CD86 in response to microvesicle treatment. Furthermore, we find that these microvesicles induce foam cell formation in macrophages, and this is enhanced by TLR activation. Microvesicles spontaneously shed by tumor cells similarly induce activation of costimulatory molecules and foam cell formation, while synthetic liposomes only induce foam cell formation. We find a common correlate of foam cell formation between tumor-shed, toxin-derived and synthetic microvesicles to be cholesterol content. Finally, foam cell formation can occur in a novel skin model of macrophage activation. These data suggest that microvesicles derived from tumors or induced by pathogens modulate local macrophage function and phenotype in vivo.

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Chloride Transport into the Neutrophil Phagosome

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Chloride is the most abundant physiological anion. The most recognized functions of this anion include regulation of cell volume, intracellular pH, fluid secretion and stabilization of resting membrane potential. However, polymorphonuclear neutrophils use this anion for a special function to produce hypochlorous acid (HOCl), the active component in bleach, for microbial killing. Myeloperoxidase (MPO), an enzyme preferentially expressed in neutrophils, catalyzes this reaction at a rate of ~134 mM/min which accounts for ~90% of oxygen consumed by neutrophils during oxygen burst. Therefore, considerable amounts of chloride are required for the biosynthesis. However, how this anion is transported to the phagosomal lumen is not fully understood. Using the self-ratioable fluorescent probe specific for chloride anion, we measured chloride dynamics within phagosomes in response to extracellular chloride changes by quantitative fluorescence microscopy. Under the experimental conditions used, normal neutrophils showed rapid phagosomal chloride uptake with an initial influx rate of 0.31 ± 0.04 mM/sec (n=5). GlyH-101, an inhibitor of the cystic fibrosis transmembrane regulator (CFTR), decreased the rate of uptake in a dose-dependent manner. Neutrophils isolated from cystic fibrosis (CF) patients showed a significantly slower rate of chloride uptake by phagosomes, having an initial influx rate of 0.043 ± 0.012 mM/sec (n=5). Interestingly, the steady state level of chloride in CF phagosomes was ~26 mM, significantly lower than that of the control (~68 mM). Because CFTR transports chloride as well as other halides, we conjugated an iodide-sensitive probe as an independent approach to confirm the results. The dynamics of iodide uptake by neutrophil phagosomes were monitored by flow

cytometry. $CFTR_{inh}172$ blocked ~40-50 % of the overall iodide uptake by phagosomes in normal neutrophils. In a parallel fashion, the level of iodide uptake by CF phagosomes was only ~20-30 % of that of the control. Taken together, these results implicate CFTR in transporting halides into the phagosomal lumen. This work was supported by the NIH grant R01AI72327.

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Active probing is required for the efficient capture of phagocytic targets by macrophages

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Binding of ligands by immunoreceptors is thought to be a passive, stochastic process. Contrary to this notion, we found that binding of IgG-opsonized particles by Fcγ receptors was inhibited by agents that interfere with actin assembly or disassembly. Changes in the lateral mobility of the receptors –assessed by single-particle tracking– or in the microelasticity of the membrane –determined by atomic-force microscopy– could not account for the effects of actin disruption on particle binding. Instead, we found that macrophages contact their targets by actively extending actin-rich structures. Formation of these protrusions is driven by Rac1 and requires phosphatidylinositol 4,5-*bis* phosphate and phosphatidylinositol 3,4,5-*tris* phosphate. Thus, macrophages continuously probe their environment for foreign particles in a manner akin to the constitutive sampling of the fluid milieu by dendritic cells. Active probing by phagocytes is most important when confronted by scarcely opsonized and/or highly mobile targets.

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TL1A drives innate and adaptive immune responses during the development of murine colitis

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TL1A is a member of the TNF superfamily that mediates a strong co-stimulation of T_H1 and T_H17 responses. The expression of TL1A and its receptor DR3 is increased in inflamed mucosa of Crohn's Disease patients and in murine models of ileitis. We have shown that in a chronic model of colitis neutralizing TL1A antibodies attenuate clinical signs of colitis by attenuating T_H1 and T_H17 responses. However, it remains to be elucidated if TL1A is essential for the development of chronic colitis.To determine if TL1A is involved in the development of colitis we utilized the DSS-induced model of colitis. To distinguish between a role for TL1A during acute colitis (induced by innate immune cells) or chronic colitis (caused by T cells) we administered either one or four cycles of DSS, respectively. $TL1A^{-/-}$ mice developed less severe acute and chronic colitis suggesting that TL1A plays an important role during the innate as well as adaptive immune response during intestinal inflammation. Less severe inflammation in $TL1A^{-/-}$ mice was accompanied with reduced IL-17 and IFN- γ production in lamina propria mononuclear cells. To confirm our data we used a T cell driven model of chronic colitis. Transfer of $CD4^{+}CD45RB^{high}$ T cells from WT mice into *Rag1*$^{-/-}$ mice resulted in the development of colitis within 4-7 weeks, as evaluated by loss of body weight, and severe intestinal inflammation. However, cohorts that received $TL1A^{-/-}$ $CD4^{+}CD45RB^{high}$ T cells did not develop colitis. We did not observe any weight loss in these mice and only very mild inflammation of the intestine. Restimulation of isolated Lamina Propria mononuclear cells or MLN derived from

mice receiving TL1A$^{-/-}$ CD4^{+}CD45RB^{high} T cells produced significantly decreased IL-17 and IFN-γ than recipients that received WT T cells. These data demonstrate that TL1A plays a crucial role in the development of chronic colitis by affecting T_H1 and T_H17 responses. Our data suggest that TL1A could be an attractive therapeutic target for chronic intestinal inflammation. NIH DK056328 (SRT).

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The mucosal phenotype and function of Dendritic Cells is regulated by PPARgamma

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Tight regulation of the mucosal immune system generally prevents harmful responses toward food antigens and commensal bacteria. Innate immune cells in the intestine are important in the first line of defense in response to intestinal antigens. Specifically, resident intestinal dendritic cells (DCs) serve a pivotal role in mucosal immune regulation via the capacity to direct antigen-specific activation and differentiation of T cells and B cells. Peroxisome proliferator-activated receptor gamma (PPAR g), a nuclear receptor family member, has been shown to have immunoregulatory functions. Therefore, we tested the hypothesis that PPAR g controls the mucosal phenotype and functions of DCs. Activation of PPAR g in murine bone marrow-derived DCs (BMDCs) increased expression of IL-10 and arginase I, reduced expression of iNOS and LPS-mediated upregulation of CD80. PPAR g activation in LPS-matured BMDCs decreased their ability to induce naïve T cell proliferation in vitro and this effect was abolished by the presence of anti-IL-10R antibody. Moreover, PPAR g activated mature BMDCs inhibited naïve B cell proliferation but promoted T cell-independent IgA production. In preliminary studies, PPAR g activated BMDCs induced CCR9 (gut-homing receptor) and FoxP3 expression, but reduced CCR5 and CCR7 expression by T cells in vitro. BMDCs from transgenic mice with a conditional deletion of PPAR g in CD11c^{+} cells (PPAR g$^{D\ DC}$) displayed increased sensitivity to LPS marked by greater upregulation of CD80 and MHCII. PPAR g$^{D\ DC}$ BMDCs had an increased ability to present ovalbumin to Ova-specific OT-II T cells and a decreased ability to induce IgA by B cells. These data indicate that the activation status of PPAR g regulates mucosal-like functions and anti-inflammatory activity by bone marrow derived DCs.

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gamma/delta T cells, monocytes and neutrophils: the innate threesome

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Activation of γδ T cells is likely to occur at early stages of infection, yet the interplay between γδ T cells and cells of the innate immune system has not been addressed in detail. Vγ9/Vδ2 T cells are a minor subset of T cells in human blood and differ from all other T cells by their immediate and MHC-non-restricted responsiveness to microbes. Given their migration properties, we believe that Vγ9/Vδ2 T cell, monocytes and neutrophils are co-recruited to the site of inflammation. Co-cultures of Vγ9/Vδ2 T cellswith autologous monocytes and/or neutrophils demonstrate that the response of Vγ9/Vδ2 T cells to bacteria depends on the capacity of these bacteria to produce the low molecular weight metabolite, HMB-PP. The interaction between the three cell types has profound consequences for the ensuing inflammatory response and the initiation of antigen-specific αβ T cell responses. Neutrophils take up and kill bacteria, and release HMB-PP into the micro-environment. Vγ9/Vδ2 T cells respond to soluble HMB-PP in the presence of monocytes and secrete inflammatory mediators, which attract further neutrophils and monocytes to the site of infection and enhance the survival of these cells. Signals received from activated Vγ9/Vδ2 T cellsinduce differentiation of monocytes into inflammatory dendritic cells, which sense TLR ligands and migrate to the draining lymph nodes where they trigger anti-microbial αβ T cell (Th1, Th17) responses. Vγ9/Vδ2 T cellsplay thus a key role

in bridging innate and adaptive immune responses in infection and inflammation. Our current work aims at investigating Vγ9/Vδ2 T cell responses in acute bacterial infection, and how their activity may affect clinical outcome. For instance, analysis of patients receiving peritoneal dialysis (PD) indicate that the HMB-PP producing capacity of the causative pathogen, together with infiltrates of activated Vγ9/Vδ2 T cells, correlate with the severity of the inflammatory response and have negative consequences for clinical outcome from episodes of acute PD-associated peritonitis. Our studies on the crosstalk of γδ T cells, monocytes and neutrophils thus have implications for early diagnosis, prognosis and treatment of acutely infected patients.

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Hypoxia increases membranal and secreted HLA-DR in endothelial cells, rendering them T cell activators

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Transplantation involves preoperative ischemic periods, which were suggested to contribute to endothelial cell (EC) dysfunction and T cell activation, leading to graft rejection. Since hypoxia is a major constituent of ischemia, we evaluated its effect on the ability of ECs to express HLA-DR, which is required for presentation of exogenous antigens to T cells, and by itself serves as an important target for allogeneic T cells. The primary human umbilical vein ECs (HUVEC) and the human endothelial cell line EaHy926 were incubated in normoxia or hypoxia (PO_2+ T cells in a HLA-DR-dependent manner, as demonstrated by the use of monoclonal anti-HLA-DR. We suggest that hypoxia post-translationally elevates ECs' expression of membranal and soluble HLA-DR, and that HLA-DR is involved in allogeneic T cell activation, explaining the pivotal role of ECs in ischemia/hypoxia-associated injury and graft rejection.

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Mechanisms of Neutrophil Priming

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Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), prime the respiratory burst of human neutrophils. Both granule exocytosis and phosphorylation of NADPH components have been implicated in this priming; however, their relative importance has not been established. We generated fusion proteins containing the TAT cell-penetrating sequence and a SNARE domain of SNAP-23 (TAT-SNAP-23) or syntaxin-4 (TAT-syntaxin-4) which inhibited granule exocytosis in a concentration-dependent manner. Priming of isolated human neutrophils was measured as the increase in fMLF-stimulated superoxide release, following incubation with 2 ng/ml TNFα for 10 min. Both TAT-SNAP-23 and TAT-syntaxin-4 inhibited secretory vesicle and specific granule exocytosis starting at concentrations of 0.6 m g/ml, with maximal inhibition being achieved at 1.0 m g/ml. Those same concentrations of TAT-

SNAP-23 and TAT-syntaxin-4 inhibited TNF a -induced priming of fMLF-stimulated superoxide release by up to 60%, without altering superoxide release in unprimed cells . TAT-SNAP-23 and TAT-syntaxin-4, at non-inhibitory concentrations of 0.4 µg/ml, synergistically inhibited granule exocytosis and TNF a priming of fMLF-stimulated superoxide release. Blocking p38 MAPK with 3 µM SB203580 inhibited TNF a-induced priming and demonstrated an additive effect with TAT-SNAP-23. Inhibition of ERK1/2 with PD098059 at 50 m M had no effect on TNF a priming but showed an additive inhibitory effect with SB203580. From these data, we conclude that priming of neutrophils for an enhanced respiratory burst involves multiple mechanisms, including exocytosis of intracellular granules and activation of p38 MAPK and ERK1/2. This work was supported by grants from the Department of Veterans Affairs Merit Review Board (KRM), the American Heart Association BGIA 0765387B (SMU), and the National Institute of Health HL087924 (SMU).

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A Mechanism by which the fine structure of LPS modulates host response

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Lipopolysaccharide (LPS), a principal membrane component in the Gram-negative bacteria, is sensed by the heterodimer complex, TLR4/MD-2. The fine structure of LPS, including the number of acyl chains and phosphate residues, varies with bacterial strain and growth condition, and is known to influence host response. Monophosphoryl lipid A (MPL), i.e., is much weaker than lipid A (LA) in triggering proinflammatory cytokine production but as potent as LA in inducing acquired immune response. MPL is a candidate for a vaccine adjuvant. Little is known, however, how TLR4/MD-2 alters the host response according to the fine structure of LPS. We here compared MPL and LA with regard to physical interaction with TLR4/MD-2, subsequent TLR4/MD-2 clustering, and activation of the downstream signaling pathways. Our results reveal a possible mechanism by which TLR4/MD-2 changes host response, and would contribute to the development of a novel type of vaccine.

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Inhibition of NFkB p65 by Suppressor of Cytokine Signaling 1 (SOCS1) within the Cell Nucleus

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Suppressor of cytokine signaling (SOCS) proteins are well known as inducible feedback inhibitors of janus kinases (Jak) and signal transducer and activator of transcription (STAT) signaling pathways. Moreover, SOCS1 is known to affect additional signalling cascades. We report the surprising observation that SOCS1, but no other members of the SOCS family, localized predominantly in the nucleus. Nuclear localization of overexpressed as well as endogenous SOCS1 was proven to depend on a novel bipartite nuclear localization signal (NLS) which could be identified in-between the SH2 region and the SOCS box. Using photoactivable SOCS1 constructs we confirmed trapid translocation from the cytoplasm to the nucleus proceeds. Within the nucleus SOCS1 was highly mobile as demonstrated by FRAP experiments. Hence we reasoned that SOCS1 features unexpected functions depending on its nuclear localization. To characterize the novel role of SOCS1 we generated NLS-mutants whose expression was restricted to the cytoplasm. By analyzing variations in their respective gene expression profiles we identified differently regulated genes depending on the nuclear availability of SOCS1. Among these we were able to identify the NFκB component p65 as interaction partner for SOCS1. SOCS1 bound to p65 within the nuclear compartment and

increased p65 ubiqutination and degradation. Binding was dependent upon the SH2 domain whereas ubiqitination was mediated through the SOCS box. Limitation of p65 resulted in reduced expression of a subset of NFκ B dependent genes. The results show that nuclear SOCS1 regulates the duration of NFκ B signaling by means of its ubiquitin ligase activity thereby exerting a so far unrecognized function.
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ADAM17 regulates neutrophil recruitment during acute lung inflammation
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The A Disintegrin And Metalloproteinase 17 (ADAM17) regulates the ectodomain shedding of various key inflammatory factors and receptors, including TNFα, its two receptors, and L-selectin, from leukocytes. To date, very little is known about ADAM17's *in vivo* role during inflammation in part because homozygous deletion of its gene is lethal. Using conditional ADAM17 knock-out mice (*ADAM17$^{flox/flox}$/Vav-Cre*), which lack leukocyte-expressed ADAM17 and are viable, we examined ADAM17's role and biological substrates in acute lung inflammation. Our ADAM17-null and control mice were exposed to LPS by aerosolization and at set time points bronchoalveolar lavage was performed and lungs harvested. We found that a deficiency in leukocyte ADAM17 resulted in a significant decrease in neutrophil recruitment to the alveolar space 8 hours after LPS exposure, the peak time point of alveolar neutrophil recruitment in our model. Interestingly, 2 hours after LPS challenge, alveolar neutrophil counts were higher in ADAM17-null mice than control mice. L-selectin directs neutrophil sequestration in the pulmonary capillaries during LPS-induced inflammation, and its surface expression was significantly increased on alveolar neutrophils from ADAM17-null mice, indicating a mechanism for their enhanced early infiltration. Alveolar levels of TNFα were greatly reduced (> 95%) in ADAM17-null mice compared to controls. Among other effects, TNFα induces the expression of chemokines that promote pulmonary neutrophil recruitment. We found that alveolar levels of LIX and KC were significantly lower in ADAM17-null mice than control mice at 2 and 8 hours, respectively, after LPS instillation. Hence, the impaired shedding of L-selectin and TNFα are potential initial mechanisms responsible for the transitory influx then marked reduction, respectively, in aveolar neutrophils in ADAM17-null mice following LPS inhalation. This study is the first to demonstrate *in vivo* that ADAM17 regulates pulmonary inflammation after exposure to LPS.
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Autophagy Suppresses IL-1b Secretion by Targeting pro-IL-1β for Degradation
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Autophagy is a key regulator of cellular homeostasis that can be activated by pathogen-associated molecules and has recently been shown to influence IL-1 β secretion by macrophages. However, the mechanisms behind this are unclear. The aim of this study was to further delineate the nature of the interactions between autophagy and the inflammasome system and here we describe a novel role for autophagy in regulating the production of inflammatory cytokines in antigen-presenting cells. After treatment of macrophages with Toll-like receptor (TLR) ligands, pro-IL-1 β was specifically sequestered into autophagosomes and further activation of autophagy with rapamycin or starvation induced the degradation

of pro-IL-1 β and blocked secretion of the mature cytokine. Inhibition of autophagy with 3-methyladenine (3-MA), wortmannin or siRNA against beclin 1 (Atg6) promoted the processing and secretion of IL-1 β by LPS-stimulated antigen-presenting cells in a NLRP3- and TRIF-dependent manner. This effect was reduced by inhibition of reactive oxygen species, but was independent of NOX2. Induction of autophagy in mice *in vivo* reduced serum levels of IL-1 β in response to challenge with LPS. These data demonstrate that autophagy controls the production of IL-1 β by targeting pro-IL-1 β for lysosomal degradation and regulating activation of the NLRP3 inflammasome. This represents a potentially pivotal role for autophagy in controlling the response to inflammatory stimuli. This work was supported by Science Foundation Ireland as part of the Immunology Research Centre, SFI Strategic Research Cluster.

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NLRP12 participates in the Host Resistance to Yersina pestis producing a Y. pseudotuberculosis-like LPS

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Yersina pestis, the causative agent of plague, is a master of immune evasion. The evasion strategies of the bacterum include generation of a tetra-acylated LPS at 37 º C, caused by the lack of lipid A acyl transferase *lpxL*. This LPS is a poor agonist for TLR4/MD-2 in innate immunity. Here we suggest that the loss of the ability to produce a hexa-acylated TLR4-activating LPS was pivotal in the evolution of high virulence in *Y. pestis* during the split from the closely related *Y. pseudotuberculosis*, which causes mild gastroenteritis. *Y. pestis* lacks *lpxL*, whereas *Y. pseudotuberculosis* contains the gene. Host resistance in mice infected with *Y. pestis* expressing *Y. pseudotuberculosis* LpxL (*Y. pestis*-pYptbLpxL) and producing a potent *Y. pseudotuberculosis*-like LPS was impacted by several inflammasome components, including NLRP12 and NLRC4. The production of IL-18 and IL-1 β was also impaired in NLRP12 and NLRC4 KO mice after infection. Importantly, IL-18 signaling appeared crucial to host resistance, and IL-1 β also contributed significantly. IL-18 is a strong inducer of IFN γ, and indeed, similar to IL-18R deficient mice, animals deficient in IFN γ R succumbed to *Y. pestis*-pYptbLpxL infection. The experiments also indicated that NLRP12, NLRC4 and IL-18R KO mice are more susceptible to wild type *Y. pestis*. To sum up, our results suggest that NLRP12 and other inflammasome components participate in resistance to *Y. pestis* generating a *Y. pseudotuberculosis* like LPS, and that an IL-18 – IFN γ axis is effective in controlling this infection. We propose a novel role of NLRP12 in the host responses to microbial pathogens.

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Mutations in the NALP3 inflammasome are associated with delayed apoptosis in human neutrophils

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We have recently described a patient with a long history of inflammatory disease resulting from excessive IL-1 β production, who was found to be a heterozygous carrier of two common polymorphisms in the genes encoding NALP3 (*NLRP3*) and TUCAN (*CARD-8*); both of which are components of the NALP3 inflammasome. This compound polymorphism was found in 4% of the general Swedish population, and the prevalence suggests a genetic predisposition for common chronic inflammatory diseases. In the present study, the spontaneous and microbe-induced apoptosis was investigated in four patients carrying the *NLRP3* (Q705K) and *CARD-8* (C10X) polymorphisms.

The apoptosis in neutrophils from both patients and matched healthy donors was investigated by Annexin V staining, and the mitochondrial damage was measured by tetramethylrhodamine ethyl ester (TMRE). The degree of spontaneous apoptosis in neutrophils was determined after 6 and 20 hours of incubation, whereas the microbe-induced apoptosis was investigated 6 hours after incubation with the bacteria *Staphylococcus aureus* . The protein levels of Mcl-1 and the phosphorylation status of Akt and ERK were investigated using Western blotting. The patients had significantly delayed spontaneous, as well as microbe-induced, apoptosis compared to controls. The patients showed both increased phosphorylation and increased levels of Akt but not ERK. Moreover, the levels of the anti-apoptotic protein Mcl-1 were increased in the patients compared to the control subjects. Blocking the activation of Akt, accelerated the spontaneous apoptosis. We find that the combined *NLRP3* and *CARD-8* polymorphisms are associated with delayed spontaneous as well as microbe-induced apoptosis. This can be explained by the finding that the patients show increased levels/phoshorylation of Akt and Mcl-1, resulting in an anti-apoptotic profile. Increased knowledge regarding the interplay between inflammasomes and apoptosomes is required to understand the regulatory mechanisms behind the apoptotic process during immunological homeostasis and inflammatory condition in these patients.

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Dimerization of TIR Domains Underlies the Immunosuppression Caused by *Brucella* TcpB and its Principle can be Used for the Design of Potent TLR Signaling Pathway Inhibitors

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OBJECTIVES: Cells participating in innate immune system respond to pathogen associated molecules by activating Toll-like receptor (TLR) signaling pathways leading to the inflammatory response. Excessive and/or inappropriate TLR signaling pathway activation can on the other hand lead to different autoimmune or chronic inflammatory disease development including sepsis, rheumatoid arthritis and systemic lupus erithematosus. TLR and TIR-domain containing adaptor interactions represent potential sites for specific TLR signaling pathway inhibition. Expression of proteins with TIR domains is used by bacteria and viruses to suppress activation of TLR signaling response to infection. RESULTS: We demonstrate that beyond containing a simple TIR domain TcpB of *Brucella* utilizes dimerization in order to block TLR activation. Surprisingly, fusion of tandem TIR domains of human TLRs and MyD88 adaptor inhibits activation, similar to the monomeric TIR domain but leads to the constitutive activation of TLR signaling pathway at overexpression. Based on the principle of TcpB we were able to design a TIR domain containing protein that has no constitutive activation and is superior to any described TIR-domain-containing protein in inhibiting TLR activation. Additionally, those properties of dimeric TIR domain proteins provide an additional insight into the molecular mechanism of intracellular TLR signaling pathway. CONCLUSIONS: We reveal the contribution of segments enabling dimerization of TIR domains for their efficient inhibition and show that principles of inhibition by bacterial TCPs can successfully be used for constructing inhibitors with TIR domains which have potential therapeutic use for treatment of inflammatory diseases.

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A gene locus in Francisella tularensis essential for capsule and LPS O-antigen biosynthesis, macrophage viability and virulence

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Francisella tularensis is a facultative intracellular pathogen and the causative agent of tularemia. Distinguishing features of *F. tularensis* are the low bioactivity of its LPS and a lifecycle that involves phagosome escape and followed by replication in the macrophage cytosol. In a recent screen for genes required for strain Schu S4 growth in human monocyte-derived macrophages (MDM) we identified a three gene locus *FTT1236-FTT1238* that is essential for synthesis of both LPS O-antigen and capsule. Mutant strains lacking functional *FTT1236*, *FTT1237* or *FTT1238* were sensitive to complement-mediated lysis and were phagocytosed more efficiently than wild-type Schu S4 in the absence of fresh serum, yet were defective for intracellular growth. Confocal and biochemical analyses revealed that this was not due to an inability to escape the phagosome or initiate replication in the cytosol. Rather, virulence was curtailed due to rapid death of infected MDM, thereby depriving these organisms of their replicative niche. In marked contrast, MDM infected with wild-type Schu S4 remained healthy for at least 32 hours post infection in spite of their high bacterial load. Complementation in *trans* restored virulence and revealed that *FTT1236* and *FTT1237* are operonic. Thus, our data not only define a gene locus required for LPS O-antigen and capsule synthesis in virulent *F. tularensis* subsp *tularensis*, but also show that surface sugars of this pathogen play a heretofore unappreciated role in virulence via their ability to prevent premature death of this pathogen's replicative niche, the macrophage. Supported by NIAID 2U54AI057160 and P01 AI044642.

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Modulation on Innate Immune Response with Various Lipid A/LPS Partial Structures; Chemical Synthesis and Elucidation of Molecular Basis

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The lipid A moiety of LPS modulates the innate immune response, and we have shown the molecular basis with various synthetic preparations. The lipid A and Kdo-lipid A from parasitic bacteria such as *Helicobacter pylori* and *Porphyromonas gingivalis* are the one of these targets. LPS of the parasitic bacteria show much lower inflammatory activity than other enterobacterial LPS such as *Escherichia coli*, and considered to be associated with chronic inflammation and atherosclerosis. The biological activities, however, have not been characterized well despite the clinical importance of these bacteria, because of the heterogeneity of the natural lipid A structures. The lipid A have distinctive structures in comparison with *E. coli* as following; they have fewer but longer acyl groups, and usually do not have the 4'-phosphate group, and the glycosyl phosphate often has an ethanolamine group (in case of *H. pylori*).

We have thus chemically synthesized *H. pylori* and *P. gingivalis* LPS partial structures focusing on the lipid A and also the peculiar acidic sugar, 3-deoxy-D-*manno*-2-octurosonic acid (Kdo) parts, to elucidate their function in the immunoregulation. In order to synthesize the various lipid A structures with a variety of acylation and phosphate patterns, we developed a strategy for comprehensive synthesis using an appropriate protected disaccharide backbone as a common intermediate. We also improved the α-selective glycosylation method for the preparation of Kdo-lipid A linkage. The biological activities of lipid A and Kdo-lipid A varied depending on the acylation patterns and the phosphate modification. Some of them showed apparent antagonistic activity against cytokine induction via NF-κB pathway but potent inducing activity to IL-18, which is considered to be associated with various inflammation. The results suggested that these parasitic bacteria regulate the immune activation with the small difference of the lipid A structures of LPS, which presumably have close relationships with their virulence.

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Structural and functional characterization of the hypostimulatory lipopolysaccharide of Granulibacter bethesdensis, an emerging pathogen in Chronic Granulomatous Disease (CGD)

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Granulibacter bethesdensis belongs to the Acetobacteraceae, a family of acetic acid bacteria previously considered non-pathogenic in humans. We found that on a per cell basis, *G. bethesdensis* was 10-100 times less effective than *Escherichia coli* at stimulating neutrophil chemiluminescence. Here, we show that cytokine secretion induced by *G. bethesdensis* in human blood *ex vivo* is similarly weak compared to *E.coli* and (through testing of blood from an IRAK4-deficient patient) that this signal passes through IRAK-4, a key signaling component of the Toll-like receptor pathway. We therefore purified the lipopolysaccharide from *G. bethesdensis* using a combination of hot-phenol extraction and gel filtration chromatography resulting in two LPS fractions, one with a laddering pattern typical of O-polysaccharide (Frac3) and another comigrating with Lipid A standards and lacking higher molecular weight polysaccharides (Frac4). Both fractions stimulated the *Limulus* amoebocyte lysate (LAL) which was used to standardize the endotoxin content among different fractions. Frac4 failed to activate the NADPH oxidase of human neutrophils compared to equivalent amounts of LAL standard endotoxin (*E.coli* O113:H10), *E.coli* O111:B4 or *Salmonella typhimurium* Re595. Furthermore, *G.bethesdensis* Frac3 or Frac4 failed to induce TNFα or IL-8 secretion in blood, even at doses 10-100 times higher than *E.coli* LPS. Composition analysis revealed the presence of a 2,3-diamino-2,3-dideoxy-D-glucose backbone instead of the typical glucosamine backbone and fatty acid analysis identified □-hydroxy residues of C14:O, C16:0 and C18:0 along with C16:0, C18:0, and C18:1. MALDI-TOF MS analysis of lipid A indicated a major ion of m/z 2169 and further structural analysis is underway. We were unable to detect dephosphorylated KDO, possibly due to the absence in the *G.bethesdensis* genome of a KdsC homologue. The unusual structure of the *G.bethesdensis* LPS may account for its relatively weak stimulation of innate immune cells and may contribute to pathogenesis.

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Repair of the Yersinia pestis palmitoyl transferase gene, pagP results in the addition of two palmitate moieties and restores a robust lipopolysaccharide proinflammatory response

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The genus Yersiniae includes the enteric pathogens, *Yersinia pseudotuberculosis* (Yps) and *Yersinia enterocolitica* (Ye), as well as *Yersinia pestis* (Yp), the causative agent of the plague. These species alternate between 37ºC, the temperature of the mammalian host, and ambient temperatures in the external environment (21ºC). This shift in growth temperature induces changes in the number and type of acyl groups on the lipid A portion of lipopolysaccharide (LPS) of all three species. One specific modification, the addition of palmitate (C16 fatty acid) is present in both Yps and Ye, but not in Yp. The addition of palmitate to lipid A requires the acyltransferase enzyme, PagP. Genomic analysis of the *pagP* gene in the different Yersiniae backgrounds identified a premature stop mutation near the end of the gene in Yp that was not present in any of the other Yersinia strains. We hypothesized that this mutation results in a non-functional enzyme being synthesized and subsequently repaired the gene sequence to that present in Yps or Ye using site directed mutagenesis.

Following gene repair in KIM and CO92 backgrounds, lipid A isolated after growth at 37ºC showed a hexa-acylated structure rather than the normal tetra-acylated structure that included the addition of two C-16 fatty acids located piggyback at the 2 and 3' position on the glucosamine backbone. Additional analytical and chromatographic analysis confirmed that the Yp PagP enzyme was responsible for the addition of both palmitate residues. *In vitro* proinflammatory responses (human macrophages or cells expressing human TLR4) using palmitoylated hexa-acylated LPS showed an increase in inflammation mediated through the TRIF/TRAM arm of the TLR4 pathway, as compared to tetra-acylated Yp LPS. Finally, *in vivo* studies in mice (BALB/cJ or mice with human TLR4-MD2 receptors)demonstrated no difference in virulence between the *pagP*-repaired or wild type Yp strains (KIM or CO92).

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Evolutionary Insights into the Innate Immune Response from the Purple Sea Urchin

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The purple sea urchin genome sequence reveals a highly complex repertoire of innate immune receptors which is unlike that of vertebrates or other well-characterized invertebrates. Nonetheless a large set of important hematopoietic and immune regulators are shared between vertebrate and sea urchin immunocytes, suggesting that the divergent protective strategies of these two deuterostome groups are integrated within conserved developmental and functional regulatory mechanisms. We approach the problem of understanding the relationships among these systems from the viewpoint of gene regulatory networks. The sea urchin embryo and larva provide a morphologically simple experimental model in which to characterize immune gene regulation in the context of an intact organism. The feeding larva has several types of immunocytes that undergo a characteristic pattern of cell migration in response to a gut-associated bacterial infection. A distinctive program of immune gene expression accompanies this response. Early after bacterial exposure, IL-17 genes are expressed transiently in the gut epithelium. Hours later immunocytes move to the gut and express a suite of regulatory and effector genes. With this simple system we are developing an immune gene regulatory model that will provide a causal framework to characterize organism-level control of gut immune reactions and the resolution of these responses. This approach will

be used to determine what aspects of the gut-associated immune system are conserved among deuterostomes and how these conserved regulatory programs interface with divergent recognition and effector mechanisms. Gene expression profiles suggest that immunity in the sea urchin larva represents a minimal version of what is present in the more complex adult. Further regulatory mechanisms are likely intercalated into the system as complexity increases in both this ontogenetic system and during evolution. This work is supported by the Canadian Institutes for Health Research and by the Natural Sciences and Engineering Research Council of Canada.

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Filopodia-localized NADPH Oxidase 4 in Regulating Metabolic Stress-induced Hyper-responsiveness of Monocyte Chemotaxis and Macrophage Recruitment

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Monocyte chemotaxis and macrophage recruitment are critical for inflammatory processes and tissue repair but also mediate atherogenesis. We previously showed that hyperlipidemia and hyperglycemia promote thiol oxidative stress, enhance monocyte responsiveness to MCP-1-induced chemotaxis *in vivo*, increase macrophage recruitment and accelerate atherosclerotic lesion formation. We now investigated the molecular mechanism involved in the hyper-responsiveness of monocyte and macrophage induced by metabolic stress. In THP-1 monocytes stimulated for 24 h with 0.1 mg/ml human LDL plus 25 mM D-glucose (LDL+HG), we observed induction of Nox4, a novel inducible NADPH oxidase we identified in monocytic cells, increased intracellular H_2O_2 levels and acceleration of MCP-1-induced chemotaxis. Adenovirus-mediated Nox4 over-expression sensitized THP-1 monocytes to MCP-1-induced cell migration, simulating the effect of metabolic stress. Conversely, siRNA-mediated Nox4 knockdown suppressed LDL+HG-induced intracellular H_2O_2 formation and accelerated chemotaxis. Confocal microscopy and immunoprecipitation studies in human monocyte-derived macrophages (HMDM) revealed that Nox4 co-localized with its dimerization partner, $p22^{phox}$, and associated with proteins involved in the formation of active focal adhesions, including phospho-FAK, and paxillin. Interestingly, Nox4 also associated with F-actin cytoskeleton in the leading edge of filopodia-like structures in HMDM and actin associated with Nox4 was *S*-glutathionylated. Diet-induced dyslipidemia and hyperglycemia in mice increased macrophage recruitment into MCP-1-loaded Matrigel plugs implanted into these mice. Macrophages from metabolically stressed mice showed a 4.9-fold increase in Nox4 expression and a 66 % increase in protein-*S*-glutathionylation compared to macrophages from control mice.

Collectively, these results suggest that Nox4-derived H_2O_2 plays a role in coordinating migration through post-translational modifications of protein thiols. In summary, our data support a novel Nox4-dependent mechanism by which metabolic disorders enhance monocyte chemotaxis and macrophage recruitment. This novel pathway is likely to contribute to the formation and progression of atherosclerotic lesions.

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MMP-9 Regulates Inflammatory Gene Expression in the Aging Left Ventricle

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Matrix metalloproteinase-9 (MMP-9) deletion results in reduced macrophage infiltration into the left ventricle (LV) and attenuates LV remodeling following myocardial infarction (MI). Aging is associated with increased macrophage numbers in the heart, and we have shown that MMP-9 levels increase in old mice. However, the effects of MMP-9 deletion on the inflammatory response to aging have not been studied. Accordingly, the objective of this study was to evaluate the effect of MMP-9 deletion on macrophage numbers and inflammatory response in the LV. Immunohistochemistry for Mac-3 showed increased macrophage content in LV of senescent wild-type (WT) mice (>25 months) compared to young (6-9 months) and old (18-22 months) mice. However, unlike post-MI, similar macrophage levels were observed in senescent WT and MMP-9 null mice. We also evaluated the expression of 84 inflammatory genes in young, old and senescent WT and MMP-9 null LV by real-time PCR. MMP-9 deletion resulted in different patterns of changes in the expression of the inflammatory markers studied. For example, the expression of interleukin-18, a pro-inflammatory cytokine produced primarily by macrophages, increased by 55% in the LV of old WT but not MMP-9 null mice. Expression of interferon-gamma (IFN-γ), a cytokine that regulate macrophage growth, activation and function, did not change with age in WT mice. Young and old MMP-9 null mice exhibited 4-fold higher IFN-γ expression than WT controls, but the difference was attenuated with age such that senescent MMP-9 null mice showed similar expression to senescent WT mice. Monocyte chemotactic protein-2 increased 5-fold and macrophage inflammatory protein-2 decreased 3-fold in senescent WT LV, and similar changes were detected in MMP-9 null mice. In conclusion, MMP-9 deletion does not alter macrophage accumulation but differentially regulates expression of inflammatory markers in the aging LV. UTHSCSA TST Program (YAC), AHA POST2150178 (RZ) and VA Merit award (MLL).

Advanced Age Alters Macrophage Polarization After Pulmonary Infection

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Advanced age induces immune system dysfunction and a significant decline in protective immunity, rendering the elderly more susceptible to respiratory tract infections and adversely impacting morbidity and mortality. Macrophages play a critical role in innate immunity, fighting pathogens and orchestrating the development of the acquired immune response. However, little is known about the combined effects of aging and pulmonary infection on macrophage polarization. Using a mouse model of intratracheal infection with *Pseudomonas aeruginosa* (PA), we compared the age-associated defects on macrophage polarization towards a pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes in young and old mice. Gross lung morphology 24 hours after infection, revealed more neutrophil infiltration, increased pulmonary edema, and thickened alveolar walls in aged mice compared to young. Additionally, a panel of M1 markers, including interleukin (IL)-6 and inducible nitric oxide synthase (iNOS) and M2 markers, including arginase I (Arg1), Ym1 and Found In Inflammatory Zone (FIZZ) 1, were analyzed. Basal IL-6 expression was lower in lungs of aged mice compared to young animals. However, relative IL-6 expression 24 hours after infection was 4-fold above baseline, regardless of age ($p<0.05$). Basal expression of iNOS was reduced in the lungs of the aged mice ($p<0.05$). There were 3-fold and 6-fold increases in the expression of Arg1 above baseline after infection in lungs from young and aged mice, respectively ($p<0.05$). Arg1 expression in lungs of aged mice was higher after PA infection than the levels found in infected, young mice ($p<0.01$).M2 late response genes, FIZZ1 and Ym1 were detectable in both age groups but expression of these genes was comparable between all groups at 24 hours. In conclusion, decreased basal IL-6 and iNOS expression and

increased Arg1 expression 24 hours post-inoculum might skew macrophage polarization towards an M2 phenotype in aged mice. (Support by NIH AG018859 and the Falk Trust.)

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S100A9, A New Potential Therapeutic Target In Chronic Inflammatory Diseases

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S100A9, a Ca^{2+}-binding protein member of the S100 protein family, is predominantly expressed by neutrophils and monocytes/macrophages. This protein acts as a damage-associated molecular pattern (DAMPs) during immune responses. Indeed, previous studies showed that S100A9 induce leukocyte activation and migration. High levels of this protein are found in serums and at inflammatory sites of patients with chronic inflammatory diseases like rheumatoid arthritis, and these levels are correlated with disease gravity. In this study, neutralizing antibodies were used in the murine collagen-induced arthritis model to investigate the importance of S100A9 in rheumatoid arthritis. Anti-S100A9 mAbs were injected twice a week starting two days before arthritis induction. Anti-S100A9 injections led to a 30% reduction of disease intensity. Cytokine production was also decreased in anti-S100A9 as exemplified by a marked reduction of IL-6. This is consistent with the fact that S100A9 induced the secretion of various cytokines like IL-6, IL-8, MCP-1, MIP-1⟨, IL-1⟨, and TNFα by human peripheral blood mononuclear cells. Interestingly, neutrophil stimulation with TNF-α led to the secretion of S100A8/A9. We conclude that S100A9 may amplify the immune response in autoimmune diseases by inducing the secretion of pro-inflammatory cytokines like TNFα which, in turn, induce the release of more S100A9, thereby producing a self-perpetuating loop. Treatment with monoclonal S100A9 may breach this amplification loop by reducing both leukocyte migration and pro-inflammatory cytokine secretion. These results indicate that S100A9 may be a potential therapeutic target in inflammatory diseases. *This study was funded by grants from the CIHR and a scholarship from the FRSQ to P.A.T.*

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Protection against Sepsis-induced Lung Injury by Selective Inhibition of Protein Kinase C- delta (δ-PKC)

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Inflammation and pro-inflammatory mediators are activators of the Protein Kinase C isotype delta (δ-PKC). In vitro, δ-PKC regulates pro-inflammatory signaling in neutrophils, endothelial, and epithelial cells, cells that can contribute to lung tissue damage associated with inflammation. In this study, a specific δ-PKC-TAT peptide inhibitor was used to test the hypothesis that inhibition of δ-PKC would attenuate lung injury in an animal model of Acute Respiratory Distress Syndrome (ARDS). Experimental ARDS was induced in rats via

cecal ligation and double puncture (2CLP), a model of polymicrobial sepsis. Following 2CLP surgery, either the δ-PKC-TAT inhibitory peptide (2CLP+ δ-PKC TAT in PBS) or PBS (2CLP+PBS) was administered intra-tracheally. Controls consisted of sham operation (SO) where animals underwent a laparotomy without 2CLP. Twenty-four hours after SO or 2CLP, blood, bronchoalveolar lavage fluid (BALF) and lung tissue were collected. 2CLP induced δ-PKC phosphorylation in the lung within 24 hours. Treatment with the δ-PKC TAT inhibitory peptide significantly decreased pulmonary δ-PKC phosphorylation indicating effective inhibition of δ-PKC activation. Plasma and BALF levels of the chemokines CINC-1 and MIP-2 were elevated in 2CLP+PBS rats as compared to SO rats. Treatment with δ-PKC TAT reduced 2CLP-induced elevations in chemokine levels in BALF and plasma, suggesting that δ-PKC modulated chemokine expression. Most importantly, intra-tracheal administration of δ-PKC TAT peptide significantly attenuated inflammatory cell infiltration, disruption of lung architecture and pulmonary edema associated with 2CLP. Thus, δ-PKC is an important regulator of proinflammatory events in the lung. Targeted inhibition of δ-PKC exerted a lung-protective effect 24 hours after 2CLP. Supported by NIH GM64552 (LEK) and GM59930 (CSD).

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Cytokine and acute phase protein mRNA expression in liver tissue from pigs with severe sepsis caused by intravenous inoculation of Staphylococcus aureus

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The aim was to substantiate previous findings of hepatic dysfunction in a porcine model of *Staphylococcus aureus* induced severe sepsis. Nine pigs were inoculated intravenously once or twice with 10^8 *S. aureus* per kilogram body weight and killed 12, 24 and 48 h later. Three pigs served as controls. Blood was sampled for bacteriology, haematology and clinical pathology. Tissues were collected at necropsy for bacteriology, gene expression analysis and histology. Bacterial counts in blood remained low, decreased in the lungs, liver and spleen, but increased in bone. All infected pigs developed sepsis characterized by fever, neutrophilia, increased serum levels of C-reactive protein (CRP) and interleukin (IL)-6, and decreased levels of serum iron. CRP and IL-6 serum levels peaked at 36 h. Serum IL-1β and tumour necrosis factor-α (TNFα) did not change. Serum aspartate aminotransferase (AST) and bilirubin were elevated at 36 and 48 h. Microabscesses were found in the livers from pigs killed at 12 h only. The livers from pigs killed at 48 h also showed light, diffuse fibrin exudation (vascular leakage). Real-time PCR showed a decreased hepatic expression of mRNA coding for albumin and increased hepatic expression of IL-6, IL-8, IL-1β, and CRP. N o increase could be detected in the IL-1α or TNFα liver-mRNA levels. IL-6, IL-8 and IL-1β expression peaked at 24 hours (2-5 fold compared to the control group). In conclusion, the increased liver cytokine mRNA levels indicate a local hepatic, non-infectious inflammatory response, and supports evidence of liver dysfunction indicated by increase in AST and bilirubin, and liver histopathology. Although hepatocyte IL-8 production has been proposed to indicate hepatocyte stress, the increased mRNA IL-8 levels, as the increase of the other cytokines, could have originated from cellular sources not constitutive to the liver. This warrants future examination of the liver by e.g. laser capture microdissection. Supported by grant no. 271-07-0417 from the Danish Medical Research Council

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ABSTRACTS

The Effect of T-cell Immunoglobulin and Mucin Domain 3 (Tim 3) Pathway Inhibition in Anti-tumor Immunity Differs According to Tumor Developmental Stage

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T cell immunoglobulin and mucin domain 3 (Tim-3) plays an important role in Th1-mediated immune response, however its role in anti-tumor immune response remains to be elucidated. In this study we wanted to know whether inhibition of Tim-3 pathway enhances the efficacy of preventive or therapeutic tumor vaccine. Using Lewis lung carcinoma (3LL) cells expressing Tim-3 extracellular domain, we showed that Tim-3 pathway inhibition increased efficacy of preventive tumor vaccine but not of therapeutic tumor vaccine. Further, we demonstrated that Tim-3 pathway inhibition in early stage of tumor development suppressed tumor growth and increased mouse survival rate. However, Tim-3 pathway inhibition did not affect frequencies of CD4+CD25+Foxp3+ T cells as well as CD11b+Gr1+ cells. Our results suggest that Tim-3 pathway inhibition may modulate anti-tumor immunity depending on the tumor developmental stage. Funding resources: a Korea Science and Engineering Foundation (KOSEF) grant (R13-2003-019) through the Chronic Inflammatory Disease Research Center (CIDRC) and the Korean Health R & D project, Ministry of Health and Welfare, Republic of Korea (A080228)

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From Bedside to Bench: A Translational Study of Blood Cells and Inflammatory Cytokines in PFAPA syndrome

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PFAPA (Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis) syndrome is a disease of unknown etiology and while rare, every paediatrician will likely encounter one case of PFAPA during their career. PFAPA primarily affects preschool children. Autoinflammatory attacks occur with remarkable periodicity every 2-8 weeks. The recurrent episodes have a major impact on the daily life of the family. At present, there are no known biomarkers for PFAPA, thus the diagnosis is based on clinical phenotype, detailed case history and the exclusion of other diseases.

To advance the pathophysiological understanding of the disease, our study tracked blood cell and serum cytokine levels during both afebrile and febrile phases of PFAPA. A cohort of ten patients (median age of 4.9 years) participated in this study. Controls were recruited among otherwise healthy children scheduled for routine surgery. Blood cells and serum cytokines were analysed by complete blood cell count (CBC) analysis and multiplex ELISA.

We identified a significant difference in absolute cell counts for five blood cell types; novel findings include increased monocytes and decreased eosinophils during the febrile episode and increased thrombocytes in the afebrile interval. Relatively modest levels of inflammatory cytokines were present in sera yet, sera concentrations of nine cytokines were distinctly different between PFAPA patients and controls.

The identification of dysregulated blood cells and serum cytokines is an initial step towards the identification of biomarkers of PFAPA disease and/or players in disease pathogenesis that will potentially improve the diagnostic process, treatment, and quality of life for children with this disease. Future investigations are required to conclusively discern which mediators are associated specifically with PFAPA syndrome.

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Combining EuroSCORE, Neutrophil Migration and Surface Markers of the Migratory Process to Better Predict Post-Operative Clinical Outcome

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Introduction & Aims

Cardiac surgery triggers the immune system, specifically the neutrophil, leading to effects on post-operative recovery. Our laboratory has demonstrated a correlation with the rate of neutrophil migration to the site of inflammation, tissue function damage and post-operative outcome. We hypothesise that an individual's neutrophil migratory potential and surface markers such as transmembrane proteins CD11b, CD99, and CD47 involved in the migratory process can be used to predict a patients post-operative clinical outcome. When used in combination with the well established EuroSCORE, we hypothesise that migratory potential and surface expression will lead to a more accurate prediction of a patient's post-operative outcome.

Method

Patients undergoing open heart surgery were recruited and consented. Neutrophils were isolated and migration assays were carried out across endothelial monolayers in response to formyl-Methionyl-Leucyl-Phenylalanine (fMLP). Surface expression of CD11b, CD47 and CD99 on isolated neutrophils was determined by Flow Cytometry with/without fMLP. Post-operative clinical parameters collected included Creatinine levels, ICU stay and hospital stay.

Results

In a cohort of 31 patients undergoing open heart surgery we found that patients can be stratified based on the ability of their neutrophils to migrate. While we found no correlation between neutrophil migratory potential and post-operative outcome, we did observe a significant correlation with neutrophil surface expression of CD47 and Day3 Creatinine levels ($p<0.05$, $r=-0.4$). We also demonstrated a significant correlation with the EuroSCORE and ICU stay ($p<0.05$). We found that the EuroSCORE alone is a good predictor of post-operative ICU stay (AUC=0.725), but when it is used in combination with pre-operative migration rates it gave a stronger AUC value of 0.732.

Conclusion

This study paves the way in identifying immunological parameters, specifically markers of neutrophil migration and function, and combining them with the EuroSCORE to better inform patient outcome, leading to improved post-operative care for at-risk patients.

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Interactions between Human Neutrophils and Porcine Endothelial Cells in the Absence of α-Gal Expression depend on ICAM-1-mediated adhesion

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The lack of suitable donor organs for transplantation is ever-increasing, with long waiting lists of patients accumulating world-wide. A significant number of those patients perish in the process. The possibility of using xenogeneic organs to bridge -over until suitable donors are identified is one of the most viable options currently available. Significant immunological, physiological and functional obstacles are still evident before xenotransplantation becomes a clinical reality. One of the initial hurdles is hyperacute rejection attributed to xenoreactive natural antibodies against the α -gal moieties and complement. Previously, we reported that innate immune cells, specifically neutrophils contribute to the hyperacute rejection process in the absence of xenoreactive natural antibodies and complement. In the present study we analyzed the interactions of human neutrophils with pig endothelial cells in which the α -gal synthesizing enzyme (1,3 galactosyltransferase) was knocked-out. We show that human naïve neutrophils were able to recognize and activate porcine endothelial cells lacking α -gal expression through calcium and reactive oxygen metabolite-dependent mechanisms. The recognition of pig endothelial cells by human neutrophils was inhibited in the presence of blocking antibodies to the adhesion molecules ICAM-1 or its counter-ligands LFA-1 α and Mac-1, on both the wild-type and the α -gal knockout endothelial cells. In summary, our data highlight the importance of innate cellular immune responses in early xenograft rejection independent of α -gal expression and open the new avenues of intervention for making xenotransplantation a clinical reality.

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Influence Of 1,25(OH)2D3 On TLR-Induced Activation Of Antigen Presenting Cells Is Dependent On The Order Of Receptor Engagement

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The active vitamin D metabolite, $1,25(OH)_2D_3$, binds the vitamin D receptor (VDR) to exert its biological effects. VDR is constitutively expressed in a number of immune cells, such as professional antigen-presenting cells (pAPCs), which constitutes macrophages (MΦ) and dendritic cells (DCs).

Vitamin D can act as a suppressor of the immune system by inhibiting IL-12 and inducing IL-10 production in DC, which is supportive of an anti-inflammatory phenotype. Recently, it was shown that activation of toll-like receptors (TLR) enhanced VDR expression in human monocytes leading to cathelicidin induction. Since potential cross-talk interactions between VDR and TLR appear to exist, we investigated how $1,25(OH)_2D_3$ administration affects TLR-induced pAPC activation.

Here, we report that the influence of $1,25(OH)_2D_3$ on TLR-induced activation of pAPC is dependent on the order of VDR and TLR engagement. Our study focuses on the effect of $1,25(OH)_2D_3$ and TLR4 agonist LPS on the production of nitric oxide (NO), and pro-inflammatory cytokines induction. Pre-treatment of pAPC with $1,25(OH)_2D_3$ inhibited LPS-induced NO production. Interestingly, these inhibitory effects were not seen when LPS and $1,25(OH)_2D_3$ were added simultaneously or when LPS preceded $1,25(OH)_2D_3$. Moreover, $1,25(OH)_2D_3$ pre-treatment of pAPCs interfered with mRNA induction of iNOS, and IL-6, but did not affect transcript levels of IL-1 β, or TNF-β. Thus, engagement of VDR by $1,25(OH)_2D_3$ inhibits TLR4 induced immune activation of pAPCs.

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Vesicle amine transport protein-1 (VAT-1) recruitment to human neutrophil membranes is dependent on phospholipase D

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Phospholipase D (PLD) is a key enzyme in the production of phosphatidic acid (PA) in neutrophils. PA has been reported to stimulate secretion, kinases and the activity of NAPDH oxidase. However PA-binding proteins that regulate these functions are still poorly characterized in human neutrophils. In this study, using phospholipid vesicles and mass spectrometry analysis, we identified 8 PA-binding proteins such as vesicle amine transport protein-1 (VAT-1), Rac2, Cdc42, RhoG, annexin III, 26S protease regulatory subunit 7, EF-1-gamma and Actin-Related Protein 2/3 complex 20 kDa subunit 4 in neutrophil cytosol. Focusing then on VAT-1 characterization, we found that N-formyl-methionyl-leucyl-phenylalanine (fMLF) induced a translocation of VAT-1 to neutrophil membranes. This recruitment was inhibited in the presence of a Src kinase inhibitor (PP2), a selective PLD inhibitor (FIPI) and primary alcohol showing that fMLF-induced PLD activity is important for the association of VAT-1 with membranes. Moreover the presence of propranolol, which inhibits PA hydrolysis, increased fMLF-mediated VAT-1 redistribution to membranes. PMA also induced the translocation of VAT-1 through a pathway dependent on PKC and PLD. Phosphorylation of VAT-1 was induced by fMLF and PMA. However this phosphorylation was only dependent on PKC after PMA stimulation suggesting that different kinases may be involved in this phenomenon. Subcellular fractionation assessed the localization of VAT-1 in cytosol, primary, secondary and tertiary granules (metallo-proteinase 9: MMP9) and in plasma membrane (CD32a) compartments in resting neutrophils. After fMLF stimulation, VAT-1 was redistributed in fractions matching MMP9 and CD32a marker. PLD1 partially overlapped with the VAT-1 fraction in resting and fMLF-stimulated neutrophils. Confocal microscopy showed that VAT-1 has an intracytoplasmic granular pattern. Colocalization of VAT-1 with lactoferrin (secondary granule marker) was partial and not enhanced after fMLF stimulation. The characterization of these PA-binding proteins is essential to better understand the role of PLD-derived PA in neutrophil functions.

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Extracellular Signal-Related Kinase and NF-κB are differentially activated by LPS and HMGB1 in a Mouse Macrophage Cell Line

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Background: The Nuclear Factor High-mobility group box 1 protein(HMGB1) and LPS have both been shown to signal through toll-like receptor-4(TLR4) to activate the immune response after injury and infection respectively. Furthermore, it is possible that endogenous danger signals(HMGB1) and pathogens(LPS) both simultaneously contribute to host response in a number of clinically relevant pathologies. The signaling pathway for LPS has been well documented, but TLR4-mediated signaling after HMGB1 stimulation remains unknown. We hypothesized that HMGB1 and LPS signaling can be differentiated by activation and location of ERK and also by NF-κB translocation to the nucleus. **Methods:** Murine macrophage-derived RAW 264.7 cells were treated with LPS(100ng/mL) or HMGB1(10μg/mL) at time points up to 2h. Cells were fixed, permeabilized, labeled with primary NF-κBp65 and pERK antibodies, Hoechst nuclear stain, and fluorescent secondary antibodies. Data was acquired using a Cellomics ArrayScan VTI HCS reader with Cytoplasm to Nucleus Translocation software. All results given are mean(% of cells with high fluorescent intensity) ± SEM, n=8. Groups comparisons were made using analysis of variance with Bonferroni Post Hoc test, α=0.05. **Results:** HMGB1 and LPS treatment

significantly activated NF-κB similarly by 15mins relative to untreated controls($p<0.01$). At 0.5, and 1h NF-κB activation was significantly elevated in LPS treated cells compared to HMGB1($p<0.01$). By 2h after treatment NF-κB was equivalently activated in both treatments. Nuclear pERK activation in LPS compared to HMGB1 treatments at 0.5h, 74.25 ± 1.73 vs. 3.15 ± 1.39 and 1h, 72.59 ± 3.17 vs. 5.31 ± 2.69 was significantly increased($p<0.001$). At 2h after stimulation, nuclear pERK in LPS and HMGB1 treatments was equivalent. Cytosolic pERK in cells treated with either LPS or HMGB1 did not increase at 0.5 or 1h after stimulation. In contrast, cytosolic pERK is significantly increased 2h after HMGB1 compared to LPS treatment, 58.33 ± 7.27 vs. 16.26 ± 3.91, respectively($p<0.01$). **Conclusions:** These data demonstrate differential activation in the timing and extent of NF-κB and ERK after LPS and HMGB1 stimulation, which suggests differential TLR4-signaling pathways to alter responses dependent on whether the signal is from a pathogen or an endogenous danger signal. Understanding these distinct signaling pathways may help future therapies to limit inflammation following trauma without suppressing important responses to infection.

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The effects of fungal cell wall structure on innate immune recognition

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Fungal infections are becoming increasingly common, with the most frequent fungal organism being Candida albicans. The molecular mechanisms for this increase in pathogenicity of C. albicans, has yet to be fully understood. For example, it still remains unknown as to why certain microorganisms present a significant pathogenic risk to humans. It has been previously hypothesized that fungal pathogens are able to mask their β-glucans within their cell wall to prevent recognition by immune system cells; therefore, we hypothesize that the opportunistic pathogen, C. albicans, conceals β-glucans within its cell wall, hindering recognition by pattern recognitions receptors (PRRs), such as Dectin-1 and toll-like receptors (TLRs) expressed by cells of the innate immune system. In this research we examined fungal cell wall structure by Transmission Electron Microscopy (TEM) and macrophage recognition of fungal pathogens by Atomic Force Microscopy (AFM). TEM analysis confirmed that C. albicans and Saccharomyces cerevisiae (non-pathogenic control) were found to have structurally different cell wall architecture. Immunolabeled sections imaged by TEM showed differences in the distribution of β-glucans of C. albicans versus S. cerevisiae. The importance of the β-glucan-Dectin-1 interaction was further examined by a series of experiments comparing unbinding forces of control and pathogenic fungal cells with macrophages using AFM. Preliminary data suggested the importance of the interaction between Dectin-1 and β-glucan in innate immune recognition of fungal pathogens.

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Anti-inflammatory activities of adenosine in human neutrophils: Gene expression profile

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Objectives. Adenosine elicits potent anti-inflammatory events in neutrophils through engagement of its A2A receptor. Knowledge of the molecular pathways that control these events remains fragmentary. We therefore studied the profile of inflammatory gene expression in

neutrophils and the impact of adenosine thereon in order to identify genes responsible for the activities attributed to adenosine.
Methods. Blood neutrophils obtained from healthy volunteers were stimulated in the absence or presence of a specific agonist of the A2A receptor, CGS 21680. Dorsal air pouches raised on wild type and A2A-ko mice were injected with LPS and exudates were collected. Gene expression was monitored using real-time PCR and ELISA was used to measure cytokine levels in cell-free supernatants.
Results. 1) Stimulation with multiple rather than single classical agonists generated a gene expression profile closer to that obtained in vivo. 2) Engagement of the A2A receptor affected the expression of a subset of these genes. Adenosine had comparable impact in vitro and in vivo.
Conclusions. Multi-pronged stimulation of neutrophils may generate gene expression profiles that are more representative of real-life situations and allowed identification of a greater number of genes under specific control of the A2A receptor. These results increase our knowledge of the anti-inflammatory potential of adenosine and should contribute to improved treatment of inflammatory conditions.
Support. This work received support from the Canadian Institutes of Health Research (CIHR grant IMH-102820 to MP). MP is a senior investigator with support from the *Fonds de la Recherche en Santé du Québec* (FRSQ).

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Evaluation of the effect of alpha-defensins human neutrophil peptides on neutrophil apoptosis

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Antimicrobial peptides (alpha- and beta-defensins, and cathelicidins) possess the potent antimicrobial activities against invading microorganisms and contribute to the innate host defense. They not only exhibit potent bactericidal activities against Gram-negative and Gram-positive bacteria but also function as immunomodulatory molecules by inducing cytokine and chemokine production, and inflammatory and immune cell activation. Neutrophil is a critical effector cell in host defense against microbial infection, and its lifespan is regulated by various pathogen- and host-derived substances. We previously revealed that human cathelicidin LL-37 and human beta-defensin (hBD)-3 suppress neutrophil apoptosis via the actions on $P2X_7$ nucleotide receptor and CC chemokine receptor (CCR) 6, respectively. Here, to further evaluate the role of human alpha-defensins in innate immunity, we investigated the action of human neutrophil peptides (HNP)-1~-3 on neutrophil apoptosis.
Neutrophil apoptosis was assessed using human blood neutrophils based on the morphological changes. Of note, HNP-1 most potently suppressed neutrophil apoptosis among HNP-1~-3, accompanied with the downregulation of truncated Bid (a proapoptotic protein), upregulation of Bcl-x_L (an antiapoptotic protein), and inhibition of mitochondrial membrane potential change and caspase 3 activity. Interestingly, a selective $P2Y_6$ antagonist MRS2578 abolished the suppression of neutrophil apoptosis induced by HNP-1 as well as UDP (a $P2Y_6$ ligand).
Collectively, these observations suggest that HNPs, especially HNP-1, can not only kill bacteria but also modulate (suppress) neutrophil apoptosis possibly via the $P2Y_6$ signaling. Considering their antiapoptotic action, antimicrobial peptides (LL-37, hBD-3 and HNP-1) are expected to exert an advantageous effect on host defense against bacterial infections by prolonging the lifespan of neutrophil, a major phagocyte engaged in the killing of invaded bacteria.

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Adenosine A2A Receptor Signaling Switches M1 Macrophages to a Novel M2-like

Phenotype

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Macrophages (M φ s) respond to activating agents in their micro- environment by adopting either a "classically" activated (M1) pro-inflammatory phenotype or "alternatively" activated (M2-like) wound healing phenotypes. M1 M φs are induced by products of pathogenic agents, such as the Toll-like Receptor-4 (TLR4) agonist lipopolysaccharide (LPS), and/or by cytokines such as interferon-γ (IFNγ). M2-like M φ s are induced by a wide range of stimuli, including interleukin-4 (IL-4), interleukin-10 (IL-10), and TGF-β . M φ s activated by these varied stimuli have been categorized as M2a, M2b and M2c subtypes. M1 M φ s express inflammatory cytokines, including TNFα and IL-12. In contrast, M2 M φ s express anti-inflammatory cytokines such as IL-10, and growth factors such as VEGF and TGF-β . Adenosine is a metabolite that rapidly accumulates extracellularly in ischemia, hypoxia and inflammation as a product of ATP breakdown. Previous work in our laboratory has shown that adenosine A_{2A} receptor ($A_{2A}R$) signaling switches Mφs from an M1 to a novel M2 phenotype. We now suggest that these M φ s be termed "M2d". M φ expression of $A_{2A}Rs$ is strongly up-regulated in response to TLR agonists, priming M φ s to switch to an M2d phenotype when extracellular adenosine levels increase. TLR agonists down-regulate phospholipase-Cβ 2 (PLCβ 2) expression in M φ s, and this down-regulation also plays a role in sensitizing M φ s to adenosine stimulation. We have characterized the expression of several canonical markers of M1 and M2 M φ s by M2d M φ s both *in vitro* and in an *in vivo* model of wound healing. This $A_{2A}R$-mediated switch of M1 M φ s to an M2d phenotype represents an attractive target for pharmacological regulation of wound healing and inflammatory diseases.

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HMGB1 induces responses distinct of LPS in macrophages.

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Objective(s): High mobility group box1(HMGB1) is a damage-associated molecular pattern(DAMP) molecule that can trigger inflammatory signaling through TLR4. However the mechanism of TLR4 activation by HMGB1 is not known. Here we carried out a comparison of HMGB1 with LPS (a prototypic TLR4 agonist) in macrophages to access the similarities and differences to gain future insight into the DAMP activity of HMGB1. Material and methods: We used human embryonic kidney(HEK) 293.7 cells, RAW 264.7 macrophages and thioglycollate-elicited peritoneal macrophages from wild-type(C57BL/6), TLR4-/-, MyD88 wild-type or MyD88 -/- mice. HEK293 cells, stably transfected with mouse control vector or TLR4, MD2 and CD14 plasmids were treated with LPS(100ng/ml) or endotoxin-free recombinant HMGB1(10μg/ml) for 18hr and NF-kB activation measured using dual luciferase assay. RAW 264.7 cells or harvested mouse peritoneal macrophages were treated similarly with LPS(0.5, 1ng/ml) or HMGB1(2,5,10μg/ml) and cytokine production was measure in supernatant by ELISA and Luminex™. Data were analyzed by Student's t-test and $p<0.05$ considered significant. Results : HMGB1 activated NF-kB significantly compared LPS(1.9 fold vs 3.2 fold, $p<0.001$) in HEK293 cells transfected with TLR4, MD2 and CD14 but not with empty control vector. Both HMGB1(2μg/ml) and LPS(0.5ng/ml) induced TNF α release from Raw 264.7 cells in dose and time dependent

maneuver. HMGB1 induced earlier significant increases in TNF α release(2hr) compared to LPS(4hr). But LPS stimulated significantly higher levels of TNF α than HMGB1 by 30hr(16669.5 vs 4331.1pg/ml, $p<0.05$). LPS induced a robust production of IL-6 (850.7pg/ml) by 45hr after stimulation. However, HMGB1 failed to induce measurable IL-6 at concentrations up to 2μg/ml after 45hr in RAW 264.7 cells. Similar levels of IP-10, KC, MCP-1 and MIP-1 α were produced by RAW 264.7 cells after LPS(0.5ng/ml) or HMGB1(2 μg/ml) stimulation. Wild-type peritoneal macrophages also produced TNF α after HMGB1 treatment. But TLR4-/- macrophages did not produce measurable TNF α even after 24hr stimulation with HMGB1(5μg/ml). Similarly, TNF α release after HMGB1 treatment from MyD88-/- macrophages was significantly attenuated compared to MyD88 wild-type macrophages(80.9pg/ml vs 971.3 pg/ml at 24hr, $p=0.005$). Conclusion: These data confirm that HMGB1 can trigger inflammatory signaling and cytokine production in a TLR4 and MyD88 dependent maneuver. We also show several similarities between TLR4 and LPS for the production of cytokines by RAW 264.7 cells. However, our results point to clean differences in the timing of the response. Also, HMGB1 fails to induce IL-6 production. Thus, there may be significant differences in the activation and signaling mechanisms for LPS and HMGB1. Delineation of these signaling pathways may be useful in the future in development of therapies to limit inflammation by endogenous danger signal leaving pathogen-initiated responses intact.

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High molecular weight hyaluronic acid (HMWHA) and low molecular weight hyaluronic acid (LMWHA) have opposite effects on fibrocyte differentiation

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Fibrocytes are monocyte derived fibroblast-like cells that play a role in both wound healing and fibrotic lesions. During tissue injury, there is an increase in extracellular matrix (ECM) turnover. Since hyaluronic acid is one of the most abundant ECM components, we examined whether hyaluronic acid affects fibrocyte differentiation. We used three different sizes of hyaluronic acid: high molecular weight hyaluronic acid (HMWHA), ~2 x 10^6 Da, low molecular weight hyaluronic acid (LMWHA), ~7.5 x 10^5 Da, and 6-mer oligo hyaluronic acid, ~1.2 x 10^3 Da. When human PBMC or purified human monocytes were cultured in the presence of HMWHA or LMWHA, HMWHA potentiated fibrocyte differentiation while LMWHA inhibited fibrocyte differentiation. However, oligo hyaluronic acid had no effect on fibrocyte differentiation. Digestion of HMWHA with hyaluronidase produced small hyaluronic acid fragments, and these digestion products inhibited fibrocyte differentiation. These results suggest that the signals required to either potentiate or inhibit fibrocyte differentiation have be a large molecule. When PBMC cultured with either HMWHA or LMWHA were stained for the hyaluronic acid receptor CD44, HMWHA increased staining for CD44 while LMWHA caused weaker staining for CD44. This indicates that not only do HMWHA and LMWHA have opposite effects on fibrocyte differentiation, but they also have opposite effects on levels of CD44. Monocytes internalize HMWHA and LMWHA equally well, suggesting that the opposite effects on fibrocyte differentiation are not due to differential internalization of HMWHA versus LMWHA. Other workers found that local LMWHA levels transiently increase immediately after injury. We hypothesize that the presence of LMWHA may then inhibit fibrocyte differentiation to transiently slow wound closure so that the repair does not take place until the injured site is clear of debris.

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Retinal Pigment Epithelial Cells mediate an Alternative Activation of Macrophages Characteristic of Myeloid Suppressor Cells

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Our previous discovery that retinal pigment epithelial cells (RPE) modulate endotoxin-induced macrophage/microglial cell functionality suggested that resident retinal macrophages might be alternatively activated. To test this possibility, we immunostained healthy mouse retinas, and laser-burned retinas. In addition, we made RPE eyecups from the same types of eyes, and used the RPE conditioned media (CM) to treat, and assay primary resting macrophages. There was uniform co-staining of Arginase1 and NOS2 in $CD11b^+$, $CD64^+$, $F4/80^+$ retinal macrophages. In contrast, the same cells were separated into two different populations in the laser-burned retina with one population $Arginase1^+$, the other $NOS2^+$, and none co-expressing the enzymes. Primary resting macrophages treated in culture with the RPE-CM from healthy retinas were induced to co-express Arginase1 and NOS2. However, different types of macrophages were seen when they were treated with RPE-CM from laser-burned eyes, POMC-knocked mice, or CM of healthy RPE with alpha-melanocyte stimulating hormone (α-MSH) or neuropeptide Y (NPY) absorbed. Resting macrophages treated with only NPY had enhanced NOS2 expression, and the macrophages treated with only α-MSH expressed Arginase1 and no NOS2. Only when the resting macrophages were treated with both α-MSH and NPY there was co-expression of Arginase1 and NOS2 with maintained high Ly6C expression. In addition, the macrophages went into apoptosis when they were treated with RPE-CM from laser-burn and POMC knocked-out mice eyes, which were diminished or without α-MSH. Adding α-MSH back into these CM prevented apoptosis. Therefore, within the healthy retina is a mechanism to induce potential suppressor macrophages, and that part of this mechanism is a process to selectively eliminate resident monocytes that cannot be immunosuppressed by α -MSH and the other mediators of ocular immune privilege. Since suppressor macrophages may hold an important role in immune homeostasis, the failure to generate these cells could potentially make the retina susceptible to autoimmune disease and unregulated wound repair.

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Suppressive Leukocytes Control Murine Ovarian Cancer Progression through IL-10 Signaling

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The ovarian cancer microenvironment is dominated by the presence of infiltrating myeloid cells that support tumor growth and suppress immune responses. Therefore, elucidating the mechanisms of their recruitment and their contributions to the tumor microenvironment is of great interest. Previous studies have shown that elimination of the CD11b+CD11c+ compartment which functions as Myeloid-Derived Suppressor Cells (MDSCs) within the murine tumor environment results in slowed tumor progression and a measurable decrease in IL-10 within the tumor ascites. The presence of high levels of IL-10 in both clinical and murine models of ovarian cancer has been well established, however the direct contribution of IL-10 to the microenvironment milieu is poorly understood. We have now identified the CD11b+CD11c+ MDSCs as the predominant IL-10 -producing cellular population in the ovarian tumor microenvironment. Importantly, blockade of IL-10 signaling with an IL-10R

antibody results in dramatically reduced tumor burden and enhanced survival. Moreover, myeloid cells in the peritoneum of antibody-treated or chimeric IL-10R-/- mice required IL-10 signaling to acquire a tumor-associated phenotype. Concomitantly, T cells from these mice show increased expression of activation markers and enhanced activity as assessed by IFN-gamma production. These studies identify a critical role for IL-10 in the development and maintenance of a hospitable microenvironment for the progression of ovarian tumors, and thereby also identify IL-10 signaling as a target for efficacious therapeutic strategies against the MDSCs that frequently inhibit the efficacy of other treatment modalities.

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Aquaporin 9 Increases Cell Adhesion and Migration in Neutrophil-like HL60 Cells

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Neutrophil migration and phagocytosis is the first line of defense in elimination of pathogens such as fungi and bacteria. These cells are the fastest migrating cells in the human body. A motile neutrophil follows a specific pattern: (i) protrusion of a broad thin lamellipodium, (ii) adhesion of the lamellipodium to the substratum and (iii) trailing of the back of the cell towards the protruding structure. Aquaporins (AQPs) are membrane-anchored water channels that are expressed in all tissues throughout the human body. Today thirteen different mammalian AQPs have been discovered and several of them have been shown to promote cell migration. In this context, we focused on the mammalian AQP9 that is known to be expressed in human leukocytes. In fibroblasts, this AQP has been shown to induce filopodia that are thin long membrane protrusions also associated with cell migration. By using various imaging techniques we here show that AQP9, when over-expressed in neutrophil-like HL60 cells, facilitates cell migration and adhesiveness. Furthermore, we propose that this mechanism might be strengthened by a localization of AQP9 to a protein complex known as the dystrophin-associated protein complex, for scaffolding and anchorage of AQP9 to membrane micro-domains. This might enable rapid polarization of the water channel during migration. Moreover, we also present a hypothetical working model for AQP9-induced membrane protrusions.

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Contribution of a Streptococcus mutans antigen expressed by a Salmonella vector vaccine in dendritic cell activation

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A *Salmonella* vector vaccine expressing the salivary binding region (SBR) of AgI/II of *Streptococcus mutans* under the control of the T7 promoter has been shown to induce a mixed Th1/Th2 anti-SBR immune response in mice and to require TLR2, TLR4 and MyD88 signaling for the induction of mucosal anti-SBR antibody responses. Since dendritic cells (DC) play critical roles in innate and adaptive immunity, the present study assessed the role of SBR expression by the vector vaccine in DC activation. Bone marrow-derived DC from wild type, TLR2, TLR4 and MyD88 knockout mice were stimulated with *Salmonella* vector BRD509, the SBR expressing *Salmonella* vector vaccine (T7), SBR protein, *Escherichia coli* LPS or Pam_3CSK_4 for 24 h. T7 and BRD509 induced upregulation of CD80, CD86, MHC II and CD40 expression. Lower levels of IL-12 and IL-10 were produced by DC stimulated with T7 than BRD509. Furthermore, T7 stimulated DC showed decreased p38, Akt and GSK-3 β

phosphorylation than DC stimulated with BRD509. However, T7 treated DC produced slightly higher levels of IL-6. The low IL-12 and high IL-6 cytokine profile expressed by T7 stimulated DC may represent a Th2-stimulatory capacity, as suggested by the increased expression in Jagged1. Moreover, the induction of IL-6, TNF- α and IL-12 production by T7 stimulated DC was more dependent on TLR4 signaling than seen with BRD509. This study demonstrates that activation of DC by the SBR expressing *Salmonella* vaccine is different from that seen with the *Salmonella* vector itself. The decreased phosphorylation of p38 and the increased expression of the Notch ligand Jagged1 in T7 stimulated DC may be involved in the Th2-skewing shift in the immune response. Moreover, SBR expression confers the *Salmonella* vector with an increased TLR4 dependence for the induction of cytokines by DC. Supported by NIH grant DE09081.

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Synthetic innate defense regulator (IDR) peptides IDR-HH2, IDR-1002 and IDR-1018 modulate various functions of human neutrophils

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Natural host defense peptides such as human β -defensins, cathelicidin LL-37 and S100A7 (psoriasin) have been reported to be not only directly antimicrobials by killing invading pathogens, but are also known to participate in numerous immunomodulatory activities by stimulating various cells, including neutrophils. Among these activities, we have previously shown that above host defense peptides caused neutrophil chemotaxis, production of cytokine/chemokines, promotion of antimicrobial functions, and regulation of apoptosis. We have recently designed a series of novel synthetic innate defense regulators (IDRs) based on natural host defense peptides, and demonstrated that both IDR-1 and IDR-1002 provide broad-spectrum protection against bacterial infections through chemokine induction. In the current work, to further assess the immunomodulatory roles of IDRs, we evaluated the effects of IDR-HH2, IDR-1002 and IDR-1018 on human neutrophils, which are effector cells in host defense. We provide evidence that IDR-HH2, IDR-1002 and IDR-1018 modulated the production of several cytokines and chemokines by neutrophils, including interleukin (IL-8), IL-10, tumor necrosis factor (TNF)- α , monocyte chemotactic protein (MCP)-1, MCP-3, and macrophage inflammatory protein (MIP)-1 α .Interestingly, these peptides selectively suppressed LPS-stimulated IL-10 and TNF- α , suggesting that IDPs likely play a role in balancing inflammation. Furthermore, IDR-HH2, IDR-1002 and IDR-1018 induced neutrophil chemotaxis, and markedly stimulated the generation of reactive oxygen species. Taken together, these observations provide novel evidence that synthetic IDRs, in addition to their anti-infective properties, possess immunomodulatory roles through their ability to activate various functions of human neutrophils.

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A-FABP deficiency in mice provides protection from tumor growth and metastasis

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Fatty acid binding proteins (FABPs) have been identified as key regulators of both metabolic and inflammatory pathways. FABPs are a family of intracellular lipid chaperones that bind various hydrophobic compounds, including long chain fatty acids and eicosanoids, and enable their diffusion within the cytoplasmic

compartment. Our studies have shown that adipocyte FABP (A-FABP) is highly expressed in macrophages where it functions to regulate cholesterol trafficking and inflammatory responses via regulation of peroxisome proliferator-activated receptor γ (PPARγ) activity. Herein, we evaluated the influence of A-FABP on tumor growth and metastasis in mice fed both normal and high fat diets. Using the LL/2 murine lung carcinoma model, we found no differences in tumor growth between wild-type (WT) and A-FABP-deficient mice fed a normal diet. However, we found significantly lower levels of lung and liver metastasis in A-FABP-deficient mice, which was accompanied by greatly reduced expression of inflammatory cytokines and matrix metalloproteinases by tumor-infiltrating macrophages (TIM). Interestingly, when WT and A-FABP-deficient mice were placed on a high fat diet, tumor growth in WT mice far exceeded that of A-FABP mice. TIM isolated from high fat-fed WT mice had significantly higher levels of IL-6, TNFa and CCL2 expression as compared to TIM isolated from A-FABP on either normal or high fat diet, or as compared with WT mice on a normal diet. These data indicate that A-FABP expression contributes to tumor metastasis and implicate A-FABP as a link between fat consumption and enhanced tumor growth and progression.

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Deficiency of Epidermal Fatty Acid-binding Protein Promotes Foxp3 expression in CD4+ T cells

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Fatty acid binding proteins (FABPs), a group of cytosolic proteins that serve as lipid chaperones, have been identified as central regulators of both inflammatory and metabolic pathways. We have shown that macrophages and dendritic cells deficient for expression of the fatty acid-binding proteins A-FABP and E-FABP have reduced inflammatory function and do not promote Th1 and Th17 responses. More recently we found that E-FABP-deficient CD4+ T cells have intrinsic defects in Th17 differentiation. This was attributed to elevated expression of peroxisome proliferator-activating receptor γ (PPARγ) in E-FABP-deficient CD4+ T cells, leading to PPARγ-mediated suppression of IL-6-induced STAT3 activity. Reduced Th17 differentiation was accompanied by elevated levels of regulatory T cells (Treg). In the present study, we demonstrate that E-FABP-deficiency promotes expression of Foxp3 expression in CD4+ T cells. Naïve CD4+ T cells from E-FABP deficient mice exhibited much higher levels of Foxp3 expression than CD4+ T cells from wild type mice when induced to differentiate into Tregs in vitro in the presence of TGF-β. Analysis of nuclear receptor expression showed elevated expression of PPARγ in E-FABP deficient Tregs, and GW9662, a PPARγ antagonist, inhibited Foxp3 expression in both wild type and E-FABP-deficient T cells. Elevated PPARγ expression in E-FABP-deficient CD4+ T cells coincided with higher levels of phosphorylated (active) AMP-activated protein kinase (AMPK), as compared to wild type CD4+ T cells. AMPK-deficient CD4+ T cells showed a decreased expression of Foxp3 when driven to Tregs in vitro. Taken together, our data indicate that E-FABP deficiency in CD4+ T cells enhances Foxp3 expression via a PPARγ/AMPK-mediated pathway.

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Targeting Chitinase-3-Like-1 Protein (CHI3L1) inhibits Breast Cancer Metastasis

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Metastasis to the lungs is one of major causes of death in breast cancer patients. Murine DA-3 mammary tumors cells metastasize to the lung 5 weeks post tumor-implantation. CHI3L1, a glycoprotein is upregulated in serum of human breast cancer patients. Serum from DA-3 mammary tumor bearers has significantly higher levels of CHI3L1 compared normals. Additionally, this molecule is upregulated in DA-3 tumor cells and in macrophages of tumor-

bearing mice. Expression of CHI3L1 in macrophages has been linked with upregulation of C-chemokine ligand 2 (CCL2) and metalloproteinase-9 (MMP-9). It is established that CCL2, a known chemoattractant for macrophages and MMP-9, a matrix degrading protein, promote tumor metastasis. Our previous studies have shown higher levels of CCL2 and MMP-9 in macrophages and T lymphocytes of mammary tumor-bearing mice. We have shown that CCL2 is involved in downregulation of IFN-γ, a crucial cytokine with anti-tumor response. Analysis of bronchioalveolar lavage (BAL) revealed higher expression of CHI3L1 in macrophages of tumor bearers compared to normal controls. Furthermore, cellular infiltrates in BAL from tumor bearing mice consisted of increased myeloid-derived suppressors, M2 macrophages ($F4/80^+$ MMR^+ cells) with decreased levels of $CD4^+$ and $CD8^+$ T lymphocytes. The composition of this type of cellular infiltrates in the lung may enhance recruitment of immunosuppressive cells that secrete TH_2 type of cytokines with decreased IFN-γ. We hypothesized that CHI3L1 expression in lungs of tumor-bearing mice affects CCL2 and MMP-9 expression in macrophages resulting in increased metastasis to the lung. Therefore inhibition of CHI3L1 is expected to decrease pulmonary metastasis via decreased CCL2, MMP-9 and increased production of IFN-γ. Our studies using chitin microparticles, a known ligand for CHI3L1, revealed that pulmonary metastasis is inhibited in the treated groups compared to the untreated controls. These studies implicate that targeting CHI3L1 improves prognosis for breast cancer via inhibition of metastasis.

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Roles for CD40 ligation in tumor-macrophage interaction and tumor metastasis.
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Macrophages are known for their ability to quickly respond to their surrounding environment. It is this adaptability that is essential for quick resolution of infection while at the same time minimizing damage to the surrounding tissue. For this process of recognition, response, and resolution of infection to occur, macrophages must be able to switch from a more pro-inflammatory state (M1) to a more anti-inflammatory state (M2).
While this ability to adapt is essential, a dysfunctional microenvironment, such as the tumor microenvironment, could manipulate macrophages to promote tumor growth while evading detection by the immune system. Although tumor-derived cytokines play a role in stimulating cytokine production by macrophages, the extent of tumor-macrophage cross-talk remains to be explored. We previously demonstrated that CD154:CD40 interactions are a dominant mechanism of contact-dependent communication between a variety of cell types. Therefore, we investigated the role of CD154:CD40 interactions in tumor-macrophage interactions. Previous studies via flow cytometry revealed CD154 expression on 3LLC carcinoma cells. In corroboration of previous studies, co-culture of supernatants of tumor cell along with normal macrophages stimulated moderate cytokine production and was equally effective with both CD40+/+ and CD40-/- macrophages. Co-culture of paraformaldehyde-fixed tumor cells with normal macrophages stimulated cytokine production by the macrophages, indicating that a cell contact dependent mechanism was also involved in the stimulation of macrophages by the tumor cells. Next, subcutaneous implantation of 3LLC tumors in both CD40+/+ and CD40-/- mice resulted in similar growth of the primary tumor mass. However, no lung metastases were detectable in the CD40-/- mice. Next, we generated bone marrow chimeric mice by reconstituting

irradiated CD40+/+ mice with either CD40+/+ or CD40-/- bone marrow. Nine weeks post-transplant the splenic and peripheral blood leukocytes were >94% donor derived. Both recipients of CD40+/+ and CD40-/- bone marrow displayed similar growth of the primary tumor and similar frequency of lung metastases, indicating that CD40 expression by bone marrow derived macrophages was not critical for tumor metastasis. The study demonstrates that ligation of CD40 on hematopoietic and non-hematopoietic cells may play critical roles in tumor progression and metastasis. This research was supported by grants from the Kentucky Lung Cancer Foundation (J.S.) and the Susan G. Komen Race for the Cure (R.S.) and by an NRSA T32 Postdoctoral Training Grant (K.R.) and a Ruth Kirschstein T31 Predoctoral Fellowship (C.G.).

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Key role of C/EBPβ and CREB in tolerogenic function of dendritic cells

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We have previously shown that bone marrow cells cultured in the presence of GM-CSF alone induced the generation of IL-10 producing tolerogenic dendritic cells (GM/DCs) whereas IL-12p70 producing DCs generated with GM-CSF and IL-4 (IL-4/DCs) are immunogenic. Molecular mechanisms involved in the generation of GM/DCs are not well characterized. In this study, we showed that GM/DCs had important C/EBP β and CREB DNA binding activities. C/EBP β is a transcription factor known to have an important role in the regulation of the cytokine production in macrophages but its expression or functions in DCs is unknown. We found that protein and phosphorylation levels of C/EBP β were similar in IL-4/DCs and GM/DCs, whereas C/EBP β DNA binding was only observed in GM/DCs. Furthermore, we found a weak DNA binding activity of CREB in IL-4/DCs as opposed to its enhanced DNA binding activity in GM/DCs. We also found that p38 MAP kinase and GSK3 played important roles in C/EBP β and CREB DNA binding activity. Indeed, inhibition of p38 activity resulted in a drastic reduction of C/EBP β and CREB DNA binding activity, drastic decrease of mRNA and production of IL-10 and increase mRNA and production of IL-12p70 by GM/DCs. In contrary, inhibition of GSK3 reduced DNA binding activity of C/EBP β but not CREB resulting enhanced maturation and increased mRNA and production IL-10 by GM/DCs. Our results demonstrate for the first time the importance of C/EBP β DNA binding in the phenotype and the cytokines production by tolerogenics dendritic cells.

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iNOS contribution to post-traumatic immunosuppression: a TLR 4 dependence in Myeloid Derived Suppressor Cells.

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Introduction/Study objective:
Nitric oxide involvement in early post-traumatic hyperinflammation is well established; recently, we have shown that nitric oxide also contributes to late immune suppression after trauma, specifically, significant splenocyte dysfunction at 48hrs after injury. We investigated the immunosuppressive cell populations in the spleen and their implication in this nitric oxide related cascade.
Methods: Wild type C57BL/6 (WT) and TLR4 knockout (KO) mice underwent either Pseudofracture(PF), a peripheral tissue trauma model, or no experimental manipulation - controls(C). **Results:** Immunofluorescence of splenic tissue identified an upregulation of iNOS

at 12 hours following trauma in WT mice when compared with controls.
Flow cytometry of spleens from WT mice in the PF group revealed changes in the proportion of splenic myeloid derived suppressor cells (MDSCs) early at 1 hour after trauma: almost a 10-fold increase in comparison with the C group. This elevated portion of MDSCs was found to be present out to 12hrs. Similarly, this change in splenic cell composition was also found in the TLR4 KO mice early following trauma.
Further flow cytometric analysis identified increased expression of iNOS within this expanded subset of MDSCs, but not until 12hrs after trauma, in comparison with the C group. (PF at 12 hrs: iNOS MFI=7407.5, vs. C: iNOS MFI=4877.11) In contrast however, TLR 4 KO mice, which showed similar expansion of MDSCs subgroups following trauma, did not express this increased level of iNOS at 12hrs after trauma. (PF at 12 hrs: iNOS MFI=6544.19, vs. C: iNOS MFI=10597.76).
This trauma-induced iNOS upregulation in MDSCs was confirmed with immunofluorescence images of cytospin slides.
Conclusions:
Nitric oxide is involved in the development of late post-traumatic immune dysfunction. MDSCs contribute to this immunosuppression through iNOS upregulation. We have identified that TLR 4 signaling is an important component for the initiation of nitric oxide mediated traumatic immune dysfunction.

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Characterization of proteins contained in exosomes during human dendritic cell maturation induced by MSU crystals
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Monosodium urate (MSU) crystals, the etiological agent of gout, have recently been described as danger signals able to induce the maturation of dendritic cells (DC) in a mouse model [1] . However, the applicability of this observation to the human system has not yet been examined. We have recently observed that MSU crystals promote the release of exosomes by myeloid cells (unpublished data). The aims of the present study were to characterize proteins contained in exosomes during MSU crystals induce DC maturation. The DC maturation process was evaluated by cytofluorometry. Exosome release was quantified by monitoring AChE activity in the cell supernatants. The proteins present in exosomes were analyzed by immuno-blotting. Our results firstly confirm that MSU crystals induce the maturation of human DC. Secondly, during MSU-induced-DC-maturation, the expression of FcγRIIa on DC was decreased to a greater extent than that that is observed during in comparison with the maturation classically induced by LPS and INFγ. Thirdly, stimulation of DC with MSU crystals increased the release of exosomes in the extracellular media and these exosomes contain FcγRIIa and also Death –associated protein-3 (DAP-3). These results point to a possible role for FcγRIIa present on these vesicles in the neutralisation of immune complexes and a possible role for DAP-3 in apoptosis induced in bystander cells. *This work was supported by grants from the CIHR (CG), NIH (PHN) and scholarships from the FRSQ and CIHR (CG).*

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Identification of a Severe Autophagy Defect in Smoker's Alveolar Macrophages
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Human alveolar macrophages play a central role in the development of smoking-related lung diseases, including emphysema/COPD. The novel hypothesis addressed in this study is that chronic cigarette smoke exposure leads to an

autophagy defect characterized by increased numbers of autophagosomes, impaired clearance of damaged proteins, accumulation of defective mitochondria and lowered pathogen clearance. Alveolar macrophages were obtained from nonsmoking and smoking volunteers. Cells from nonsmoking individuals were cultured with and without cigarette smoke extract (CSE) and cells from smokers were studied at baseline. Autophagy vesicle formation was studied using the autophagy marker, LC3-II, and transmission electron microscopy. Autophagy function was evaluated by studying protein aggregate clearance (polyQ-luciferase vector and ubiquitin complex analysis), mitochondrial health (JC-1 and microscopy) and delivery of pathogens to lysosome (FITC labelled bacteria and Lysotracker staining). The experiments performed on both normal CSE exposed alveolar macrophages and smoker's macrophages found the following smoking related changes: **1.** Increases in the number and size of autophagosomes. **2.** Decreased clearance of aggregated proteins. **3.** Accumulation of dysfunctional mitochondria. **4.** Impaired trafficking of bacteria to lysosomes. In these studies, smoking-induced changes in the process of autophagy led to an accumulation of autophagic vesicles and a decrease in autophagy clearance functions. The autophagy/lysosomal degradative pathway has a number of known pharmaceutical interventions. The identification of an autophagy defect in alveolar macrophages from smokers suggests their possible efficacy in smoking-related lung diseases. FUNDING SOURCE :NIH R01 HL079901, NIH RO1 HL079901 and NCRR UL1 RR024979-03S4 to MM and NCRR UL1 RR024979-03S4, NCRR KL2RR024980 to AG and SH. U. of Iowa, NCRR UL1RR024979. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

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The Tissue Kallikrein Family: Expression of New Members in the Human Neutrophil

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The human tissue kallikrein family comprises a group of 15 serine proteases [HK1 (*KLK1*) to HK15 (*KLK15*)] that has a differential distribution in cells and biological fluids. The most studied tissue kallikrein (HK1), is a kininogenase so termed because it has the capacity to release the kinin peptides from endogenous protein substrates called kininogens. Kinins are proinflammatory peptides that modulate neutrophil activity by activating the kinin B1 receptor. Other members such as HK6 and HK13 are associated to degradation of extracellular matrix components. The current study was designed to elucidate whether other members of the tissue kallikrein family, apart of HK1 are expressed in human neutrophils.

Neutrophils, isolated from peripheral blood of clinically healthy donors were used to determine the expression of the fifteen members of the family. When neutrophil homogenates were analyzed by Western blot, immunoreactive HK12 was absent, whereas the remaining members were present and displayed the expected molecular mass that ranged between 25 to 70 kDa. By contrast, conventional RT-PCR using well characterized primers showed that mRNA expression was restricted to the occurrence of *KLK1*, *KLK4*, *KLK10*, *KLK14* and *KLK15* genes indicating that immunoreactive protein levels do not necessarily correspond with the spectrum of mRNAs. With brightfield immunocytochemistry HK1, HK2, HK3 and HK13 kallikreins showed an intracellular cytoplasmic granular pattern whereas HK4, HK6 and HK10 have a preferential distribution of immunoreactivity on the neutrophil cell membrane. In support, protein structure analysis of the various kallikreins predicted the existence of transmembrane domains for HK4 (residues 7 to 24), HK5 (residues 7 to 29) and HK8.

Our results demonstrate for the first time the expression of fourteen members (HK2 to HK15) of the tissue kallikrein family, apart from HK1, in the human neutrophil, and suggest that they may enhance neutrophil activity as well as influence microenvironment components in inflammatory disorders.

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Phagolysosomal fusion is insufficient for inhibiting M. tuberculosis growth in human macrophages

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The best characterized virulence mechanism of *Mycobacterium tuberculosis* is the inhibition of phagolysosomal fusion, which creates a niche allowing for bacterial replication. However, studies have shown that *M. tuberculosis* can escape into the cytoplasm; a suggested prerequisite for replication. Although the ability of LPS/IFN-γ-stimulated mouse macrophages to reduce *M. tuberculosis* replication is well-known, this is unclear for human cells. We studied the relationship between phagolysosomal fusion and bacterial replication in *M. tuberculosis*-infected human monocyte-derived macrophages. Through staining of lysosomal markers, we observed the kinetics of phagolysosomal fusion in macrophages containing H37Rv or H37Ra, and found that both reduce phagolysosomal fusion. Subsequently, we showed that increased presence of lysosomal markers on the phagosome does not *per se* mean that bacterial replication is inhibited. Furthermore, we established that IFN-γ treatment of human macrophages did not inhibit intracellular growth of H37Rv. By transmission electron microscopy, we found *M. tuberculosis* bacilli which could be interpreted as extraphagosomal. We suggest that the ability of human macrophages to counteract the immune evasion strategies of *M. tuberculosis* is not simply dependent on delivery of lysosomal components to the phagosome, and that human and mouse macrophages use different pathways to inhibit *M. tuberculosis* growth.

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The c-Cbl/CIN85 pathway mediates the downregulation of FcγRIIa in human neutrophils in a PKC-dependent manner

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Little is known about the mechanisms that arrest Fc γ RIIa signalling in human neutrophils once engaged by immune complexes or opsonised pathogens. Here we report on the mechanisms involved in this event. We first observed a loss of immunoreactivity of FcγRIIa by immuno-blotting following its cross-linking and, by flow cytometry, a stimulated internalisation of FcγRIIa leading to the down-regulation of its surface expression. Immunoprecipitation of the receptor showed that FcγRIIa is ubiquitinated after stimulation. MG132 and clasto-lactacystin β-lactone inhibited the loss of immunoreactivity of FcγRIIa, suggesting that this receptor was down-regulated via the proteasomal pathway. The E3 ligase c-Cbl and the adaptor protein CIN85 were found to translocate from the cytosol to the plasma membrane following receptor cross-linking. c-Cbl and CIN85 were also recruited to the same subset of high-density detergent-resistant membrane fractions in which stimulated FcγRIIa partitioned. Silencing the expression of c-Cbl and CIN85 by siRNA in db-cAMP-differentiated PLB 985 cells decreased FcγRIIa ubiquitination and prevented its degradation. This pathway appears to be positively regulated by classical PKCs as FcγRIIa degradation was inhibited by the classical PKC inhibitor Go6976. We conclude

that c-Cbl mediates the ubiquitination and degradation of stimulated FcγRIIa *via* the proteosomal pathway. This event is regulated by the adaptor protein CIN85 in a PKC-dependent manner and contributes to the termination of FcγRIIa signalling.

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Disruption of Host Macrophage Small GTPase Signalling by Mycobacterial Nucleoside Diphosphate Kinase

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Background: Microorganisms capable of surviving within macrophages are rare, but represent very successful pathogens. One of them is Mycobacterium tuberculosis (Mtb) whose resistance to early mechanisms of macrophage killing and failure of its phagosomes to fuse with lysosomes causes tuberculosis (TB) disease in humans. Thus, defining the mechanisms of phagosome maturation arrest and identifying mycobacterial factors responsible for it are key to rational design of novel drugs for the treatment of TB. Previous studies have shown that Mtb and the related vaccine strain, *M. bovis* bacilli Calmette-Guerin (BCG), disrupt the normal function of host Rab5 and Rab7, two small GTPases that are instrumental in the control of phagosome fusion with early endosomes and late endosomes/lysosomes respectively. Previous work from our lab suggested that BCG secretes GTPase activating protein (GAP) activities that inactivates Rab7, leading to exclusion of an essential downstream effector, RILP (Rab7 interacting lysosomal protein) for proper phago-lysosome fusion.
Methodology/Principal Findings: We hypothesized that secreted Mtb nucleoside diphosphate kinase (Ndk) has the potential to express GAP activities towards Rab GTPase. First, in *vitro* cell-free system studies revealed to us that recombinant Mtb Ndk, but not Ndk from *M. smegmatis*, a non-pathogenic species, exhibits GAP activity towards both Rab5 and Rab7, thereby inducing the switch from Rab-GTP molecule into inactive Rab-GDP form. Thereafter, using a model of protein-coated latex beads, we demonstrated that Ndk inhibits fusion of bead-containing phagosomes with lysosomes in the macrophage. Maturation arrest of phagosomes containing Ndk-beads was associated with the inactivation of both Rab5 and Rab7 as evidenced by the lack of recruitment of their respective effectors EEA1 (early endosome antigen 1) and RILP. Consistent with these findings, macrophage infected with BCG expressing anti-sense mRNA to Ndk resulted in increased fusion of its phagosome with lysosomes along with decreased intracellular survival of the mutant.
Conclusion: Our findings provide strong evidence that mycobacterial Ndk is a putative virulence factor that inhibits phagosome maturation and promotes survival of mycobacteria within the macrophage through disruption of host small GTPase activation and trafficking.
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An inducible mechanism of resistance to lysosome damage in macrophages

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In addition to the lysosome's important roles in digestion, antigen processing and microbial destruction, lysosome damage in macrophages can trigger cell death and release of the inflammatory cytokine IL-1β. To examine the relationship between endocytosis, lysosome damage and subsequent events such as caspase-1 activation and IL-1β secretion, we developed a method for measuring lysosomal disruption inside individual living cells, which quantifies release of fluorescein-dextran from lysosomes. This novel method used fluorescence microscopy to measure the pH and therefore the compartmental location of lysosomal fluorescein-dextran. Unperturbed, cultured, murine bone marrow-derived macrophages exhibited low levels of lysosome damage.

Lysosome damage following phagocytosis differed with different types of ingested particles, with negligible damage after ingestion of sheep red-blood cell ghosts, intermediate levels by polystyrene beads, and high levels of damage by ground silica or silica microspheres. Activation of caspase-1 and subsequent release of IL-1β was proportional to lysosome damage following phagocytosis. The low level of damage following polystyrene bead phagocytosis was insufficient to activate caspase-1 in LPS-activated macrophages. These results indicated that lysosome damage following phagocytosis is dependent on particle composition and dose and that caspase-1 activation and IL-1β secretion correlate with the extent of lysosome damage. Pretreatment of macrophages with LPS, peptidoglycan, tumor necrosis factor-α, or interferon-γ decreased the amount of lysosome damage following phagocytosis of polystyrene beads, silica microspheres and ground silica. The LPS dependent resistance to lysosome damage was partially dependent on MyD88, but the resistance induced by TNF-α was MyD88 independent. These studies indicate that murine macrophages possess mechanisms induced by a variety of exogenous mediators which either increase lysosomes resistance to damage or induce a mechanism for repairing damaged lysosomes.

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PI3K p110α Regulates Late Endosomal Trafficking and Phagosome Maturation

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Phosphatidylinositol 3- kinases (PI3Ks) are lipid kinases that catalyze the phosphorylation of the 3'-hydroxyl group of phosphatidylinositol and phosphatidylinositides (PIs). PIs regulate diverse cellular signaling pathways affecting functions as diverse as cellular metabolism, cytoskeletal dynamics, and vesicle trafficking amongst others. One PI in particular, phosphatidylinositol 3-phosphate (PI3P), has been shown to play an important role in regulating the recruitment of various effector proteins essential to phagosome maturation. Although all three classes of PI3Ks can either directly or indirectly produce PI3P, only the class III enzyme Vps34, has been implicated in phagosome maturation. Previous work in our laboratory demonstrated that vitamin D_3, a known activator of class IA PI3Ks, can rescue the phagosome maturation block induced by Mtb lipoarabinomannan, indicating that this class of PI3K isoforms may also be involved in regulating phagosome maturation. To examine the contribution of class IA PI3Ks to endosomal trafficking and phagosome maturation, we studied phagosome maturation in a human monocytic cell line in which expression of the p110α isoform was knocked down using RNAi. This cell line displays a ~70% reduction in expression of p110α when compared to control cells. Cells were fed magnetic beads, and phagosomes were isolated. Flow organellometry was done to determine acquisition of early phagosome maturation markers Rab5 and EEA-1, as well as the late maturation markers Rab7, cathepsin D, and LAMP-1. Phagosomes from p110α knockdown cells displayed a significant decrease in acquisition of the lysosomal marker LAMP-1. In addition, phagosomal acquisition of the lysosomal hydrolase, β-galactosidase, was also significantly decreased, and this was found to be true irrespective of the prey utilized (either beads or *Mycobacterium smegmatis*). These results suggest that the class IA PI3K isoform p110α plays a role in late endosomal/lysosomal trafficking and phagosome maturation. Studies are currently underway to delineate the mechanism by which this class of PI3K acts on vesicular trafficking in macrophages.

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Interaction of the Mycobacterial Lipoamide Dehydrogenase with components of the Macrophage Endosomal System

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Studies from our laboratory have demonstrated that mycobacteria secrete mycobacterial lipoamid dehydrogenase (LpdC) within the macrophage and that LpdC translocates to the phagosomal membrane where it physically interacts with host actin-binding protein coronin-1. Aberrant and prolonged retention of coronin-1 on the phagosomal membrane was shown to contribute significantly to phagosome maturation arrest, which ultimately allows survival and persistence of pathogenic mycobacteria within the macrophage.. Defining how LpdC retains coronin-1 on the phagosome is therefore critical to understanding Mtb pathogenesis mechanisms.
We are currently investigating LpdC's ability to bind to phosphatidylinositol phosphates (PIPs). PIPs play important roles in the cells including cell signaling, membrane trafficking, and phagosome maturation and it is previously known that pathogenic bacteria employ different strategies to interfere with the host PIP metabolism. For example, *Legionella pneumophila* uses PIPs as membrane anchor through the production of PIP binding proteins and *Shigella flexineri* produces PIP metabolizing enzymes that directly modulate the host cell PIP levels. The interaction between PIP and LpdC therefore is potentially important in Mtb pathogensis.
We performed protein lipid overlay (PLO) assay and found that LpdC bind to a variety of PIPs with the highest affinity to PI3P. We then generated LpdC fragment proteins covering the full length of LpdC and identified the PI3P binding site. PI3P recruits specific downstream effector proteins and regulate trafficking pathways through proteins such as EEA1 and p40phox. EEA1 is an important regulator of vesicle fusion and promoter of phagosome maturation while p40phox is an essential component of the NADPH oxidase (NOX). We therefore hypothesize that LpdC inhibits phagosome maturation by competing with EEA1 for PI3P and inhibits NOX assembly by interfering with PI3P-p40phox interaction. We are also investigating whether the PI3P binding domain of LpdC overlaps the coronin binding domain and this will help us understand whether PI3P play a role in coronin-LpdC interactions on the phagosome membrane.
These key domains in the LpdC molecule that alter phagolsysosome fusion can be proposed as potential drug target.

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Intracellular sorting of chitinase-like protein SI-CLP requires interaction of GGAs with novel binding motif in the cytoplasmic tail of stabilin-1

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Macrophages are major source of secreted chitinase-like protein that combine both growth factor and cytokine functions and serve as biomarkers for chronic inflammation and carcinogenesis. The lysosomal chitinase-like protein SI-CLP has been identified by us as intracellular ligand for stabilin-1. Stabilin-1 is a unique scavenger receptor that combines endocytic and intracellular sorting functions in macrophages. We showed, that Stabilin-1 mediates sorting of newly synthesized SI-CLP from TGN to the lysosomal secretory pathway.- A DDSLL motif in the cytoplamsic tail of stabilin-1 interacts with GGA adaptors; however deletion of DDSLL reduces but does not abrogate this interaction. Here we identified a novel GGA-binding site EDDADDD in the cytoplasmic tail of stabilin-1. Deletion of EDDADDD impaired, and deletion of both DDSLL and EDDADDD sites abrogated interaction of stabilin-1 with GGAs. Surface exposure of stabilin-1 and stabilin-1-mediated endocytosis of acLDL, SPARC, and PL were neither affected by the deletion of DDSLL or EDDADDD nor by the deletion of both. At the same time both GGA-binding sites were

necessary for the intracellular sorting of SI-CLP performed by stabilin-1. Our data indicate that novel GGA-binding site EDDADDD is essential for stabilin-1-mediated intracellular sorting , but is not required for endocytosis. In conclusion, stabilin-1 requires co-operative action of both GGA-binding sites for targeting of SI-CLP to the secretory lysosomal pathway in alternatively activated macrophages

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Ion Transport and Myeloperoxidase-mediated Oxidation in Phagolysosomes Isolated from Human Neutrophils

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A method is described for rapid, high yield and functional isolation of phagosomes from human neutrophils. Paramagnetic latex microspheres were opsonized with antigen-antibody complexes and C3b, and phagocytozed by the cells. After homogenization by nitrogen cavitation, the bead-laden phagosomes were isolated magnetically. The phagosomes recovered were relatively free of cytosolic contaminants. Specific protein markers associated with this organelle such as lysosomal-associated membrane protein-1 (LAMP-1), myelo-peroxidase (MPO) and lactoferrin (LF) were enriched ~13.1, ~4.6 and ~7.6 fold, respectively. When FITC-dextran was included in the phagocytosis medium, more than half of the isolated phagosomes retained the fluorescent label after isolation, indicative of complete membrane enclosure. The isolated phagosomes showed a robust NADPH oxidase activity and were capable of acidifying via the vacuolar-ATPase (V-ATPase) proton pump. The acidification was facilitated by chloride co-transport stimulated by a cAMP anolog, which involves the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. The MPO oxidation system was able to iodinate the proteins covalently conjugated to the phagocytosed beads. Uptake of radioactive iodide by the isolated phagosomes and the subsequent MPO-mediated protein iodination was blocked by chloride channel inhibitors, including the CFTR-specific inhibitor CFTR(inh)-172. The functional inhibition by CFTR(inh)-172, together with the demonstration of CFTR physical association with isolated phagosomes by immunostaining and immuno-blotting, supports the hypothesis that CFTR plays an important role in the transport of halides into the phagosomal lumen. The phagosomal preparations can be obtained in less than one hour, retain critical ion transport and enzymatic functions, and thus, should be valuable for future functional studies. This work was supported by the NIH-R01AI72327 grant to GW. This work is dedicated to the memory of our coworker and friend, Martha L. Aiken, who died prior to its completion.

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A simple rapid method for the isolation of neutrophils from mouse bone marrow or blood using a rapid, column-free negative enrichment method that does not require density centrifugation

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Neutrophils are phagocytic leukocytes that act as a first line of defense against infection. Their main function is to eliminate deleterious agents, however they also play a role in inflammatory diseases such as rheumatoid arthritis. In mouse, neutrophils have been defined by their Gr-1 expression. GR-1 reacts with a common epitope on both Ly6G and Ly6C antigens. Recently it has been shown that inflammatory mouse monocytes share the GR-1 antigen through expression of Ly6C, but not Ly6G. Therefore it is important that neutrophils be distinguished

from other cell types when assessing cell function. Although murine models are extensively used in the investigation of various inflammatory diseases, a simple, reliable method by which viable neutrophils can be isolated has not been developed. Here we describe an easy and rapid method for the enrichment of neutrophils from mouse BM or peripheral blood that does not require a density gradient and yields highly purified, viable cells. BM was harvested from femurs and tibia by crushing the bones. Blood was collected with heparin and red blood cells were removed by ammonium chloride lysis. The neutrophils were then enriched using immunomagnetic, column-free negative selection (EasySep®). Briefly, unwanted cells were specifically labeled with dextran-coated magnetic particles using a cocktail of bi-specific tetrameric complexes. The sample was placed in a magnet and the supernatant containing untouched neutrophils was collected. Purity of $CD11b^{+}Ly6G^{+}$ cells, as assessed by flow cytometry, was 84±5% from BM (n=23) and 85±5% from blood (n=3). On average, $1x10^{7}$ neutrophils were recovered from $1x10^{8}$ total BM start cells, while $4x10^{5}$ neutrophils were recovered from $1x10^{7}$ total start lysed blood cells (or approximately $2x10^{5}$ $CD11b^{+}Ly6G^{+}$ cells per mL of blood). This protocol will provide easy access to BM or blood neutrophils for further study of immune and inflammatory responses.

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Rac Regulation of NADPH Oxidase in Phagocytic Leukocytes: More to the Story than p67phox.

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In the superoxide-generating NADPH oxidase (NOX) family, NOX 1,2, and 3 have been shown to require the Rho GTPase Rac for oxidase activation. Interestingly, a Rac binding site was identified and found only in these 3 NOX members in a recent study, where NADPH oxidase activation in human neutrophils was found severely impaired when Rac-Nox2 interaction was blocked with a TAT-peptide(*J Biol Chem*, 2008, 283,12736-46). In the current study, we expressed a NOX2 with a point mutation Tyr 425Ala in the Rac binding site in a human promyelocytic cell line lacking endogenous NOX2. Superoxide production by the mutant expressing granulocytes stimulated with either phorbol ester, fMLP, IgG-opsonized latex beads, or serum-opsonized zymosan, was severely impaired. Assembly of the NADPH oxidase on plasma membrane, including Rac translocation, appeared normal in NOX2 mutant – expressing cells stimulated with PMA. However, translocation of Rac to phagosome membrane was decreased in the mutant cells, suggesting a possible supporting role for Rac-NOX2 interaction in recruiting or retaining Rac on phagosome membrane. Analysis of INT reduction in permeabilized PLB-985 granulocytes showed that electron transfer from cytosolic NADPH to FAD on the mutant Nox 2 was drastically reduced, consistent with a regulatory role of Rac in this initial step of electron transfer for superoxide generation. Additionally, the defective oxidase activity could not be rescued by expression of either a constitutively active Rac1Q61L or overexpression of WT Rac1 in COS^{Phox} cells expressing this mutant NOX2. Taken together, this study supports a model previously proposed for Rac participation in each of the two electron transfer steps through flavocytochrome *b*(Diebold and Bokoch, *Nature Immunology*, 2000, 2, 211-5), and provides additional evidence that Rac regulates NADPH oxidase activation in phagocytic leukocytes through a direct interaction with NOX2 in addition to its interaction with $p67^{phox}$.

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Impact of Slc11a1 on long term exposure to cigarette smoke for the development of bladder cancer

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Bladder cancer is the fourth most common cancer in men in the United States. Cigarette smoking is thought to contribute to bladder cancer. However, the etiology of bladder cancer and how cigarette smoke causes bladder cancer remains unknown. While aryl amine carcinogens such as 2-naphthylamine and 4-aminobiphenyl; in cigarette smoke pass from the lungs into the blood and are concentrated and excreted by the kidneys into the bladder, nicotine and other small molecule enhancing agents in cigarette smoke quickly cross the blood – brain barrier and stimulates the release of many chemical messengers and neurotransmitters such as dopamine contained within synaptic vesicles. Our hypothesis is that Slc11a1 is a key determinant of cigarette smoke - nicotine addiction dependent bladder cancer development. Nicotine mediates its effects by acting on nicotine acetylcholine receptors in the brain. Smoking reduces upregulation of gamma - aminobutryic acid receptor (GABAA) receptors which function in inhibitory synapses. Genetic linkage studies of more than 600 families of smokers with nicotine addiction demonstrated linkage with GABA-B receptor subunit 2 (GABAB-2) and the GABA receptor associated protein (GABARAP) genes. GABARAP belong to a new microtubule associated protein (MAP) family, encoding very similar proteins, which participate in complex clustering, targeting and degradation of GABAA receptors on post synaptic membranes of neurons. Thus, cumulative effect of long-term exposure to environmental risk factors, coupled with inherited genetic and epigenetic factors which are associated with nicotine addiction should increase the risk of development of bladder cancer. Bone marrow derived macrophages were made by continuous culture in MCSF for 7 days and thereafter stimulated with nothing or with the TLR-2 agonist Pam 3CSK4 for 8 hours. Global gene expression profiling was performed using the Illumina mouse WG-6 v2.0 beadchip microarray. Basal levels of post synaptic receptors such as gamma – aminobutyric acid receptor subunit alpha 4 (Gabra4), gamma-aminobutryic acid receptor subunit gamma 1, (Gabrg1), cholinergic receptor muscarinic 4 (Chrm4), cholinergic receptor muscarinic 3 (Chrm3), neuronal acetylcholine receptor subunit 2 (Chrnb2), and the glycine receptor alpha 4 (Glra4) were higher in Slc11a1+/+ BMDMs. In addition, basal levels of exosome and neuronal lysosomal genes were higher in Slc11a1 -/- BMDMs whereas levels of synaptic vesicles and releasers of neurotransmitters were lower in Slc11a1 -/- BMDMs. Following treatment with the TLR2 agonist Pam3CSK4, there was down regulation of expression of these inhibitory synaptic receptors in Slc11a1 +/+. However, treatment with Pam3CSK4 had no significant effect on Slc11a1-/- BMDMs which were already low at basal levels. Of note, is that the basal level of GABA receptors and the GABA receptor associated proteins (Gabarap, Gabarapl1 and Gabarapl2) are inversely correlated. Whereas, the basal level of Gabarap and GABA receptor associated protein like 1 (Gabarapl1) were higher in Slc11a1-/- than in Slc11a1 +/+ BMDMs the reverse is the case for GABA receptors.

We provide preliminary data to suggest a role for Slc11a1 in small molecule - nicotine addiction dependent bladder cancer development.

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Glycine-induced Enhancement of Neutrophil Bactericidal Activity

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Compromised neutrophil functions are frequently observed in severe bacterial infections. Enhancement of the microbicidal activity of neutrophils could complement a direct antimicrobial therapy. Recently we reported that lysophosphatidylcholine (LPC), an endogenous lipid, enhances neutrophil bactericidal activity via Gly·GlyRα2/TRPM2/p38 MAPK signaling pathway (Hong et al. 2010, J. Immunol. 184: 4401-4413). However, the direct effect of glycine on neutrophil bactericidal activity was not studied. In the present study, we examined the effect of glycine on neutrophil bactericidal activity. Glycine enhanced bactericidal activity in human neutrophils and increased bacterial clearance in mice. Glycine increased TRPM2-mediated [Ca2+]i increase in neutrophils that had phagocytosed E. coli. Glycine enhanced Lucifer yellow uptake (fluid-phase pinocytosis) and azurophil granule-phagosome fusion in a p38 MAPK inhibitor SB203580-sensitive manner. These results show that activation of GlyRα2 enhances neutrophil bactericidal activity by increasing azurophil granule-phagosome fusion via stimulation of TRPM2-mediated calcium/p38 MAPK signaling. We propose neutrophil GlyRα2 as a novel target for anti-microbial therapy.

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The short chain fatty acid sodium butyrate regulates the induction of CD1a in developing dendritic cells

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Dendritic cells (DCs) are professional antigen-presenting cells with attributes for priming/activating T cells and mediating immune responses. Considering the importance of DCs in the initiation of immune responses, it will be of interest to study their mechanisms of regulation. Histone-modifying enzymes, such as histone deacetylases (HDACs), are critical in controlling chromatin organization. The aim of our study was to investigate DC differentiation and activation under the influence of sodium butyrate (NaB), a short chain fatty acid that is a histone deacetylase inhibitor. Monocytes from healthy individuals were differentiated into immature DCs with IL-4 and GM-CSF in the presence or absence of NaB. DC differentiation was evaluated by CD14 and CD1a expression by flow cytometry. We observed that monocytes stimulated to differentiate in the presence of NaB displayed colony formation and dendritic cell morphology, lost CD14 and showed decreased secretion of IL-1beta. The acquisition of CD1a, however, was impaired. Being a natural short chain fatty acid, NaB may regulate CD1a acquisition independently of its HDAC inhibitory activity. We observed that the addition of peroxisome proliferator-activated receptor gamma (PPAR- gamma) antagonist (GW9662) did not reverse NaB effect, suggesting this was not the pathway involved. On the other hand, CD1a can also be induced by toll like receptors 2 (TLR 2) agonists, such as Pam3Cys, and NaB inhibited this effect. Our data suggest that the histone deacetylase inhibitor NaB instead of impairing DC differentiation inhibits the acquisition of CD1a induced both by cytokines and by TLR 2 agonist stimulus. Furthermore, this occurs at the transcriptional level as NaB led to a decrease in mRNA levels of CD1a.

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Toll-Like Receptor Phenotyping/Functional Analysis: Dendritic Cells and Cell Line Model Systems for Immune/Inflammatory Signaling

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TLR phenotyping of Dendritic Cells (DC) by Flow Cytometry has been developed into a 4 color Flow Cytometry assay to characterize DC subsets. Combinations of TLR specific antibodies, lineage markers and DC subset markers such as CD123 for plasmacytoid DCs provide a standardized DC subset phenotyping system with flexibility for in depth analysis.

Extension of the Flow analysis by measurement of intracellular staining of induced cytokines or other inflammatory mediators enables functional analysis of DC subsets and their response pathways which direct $CD4^+$ cell polarizing responses into Th17, Th1 or Th2 cells.

TLR/ NF- κB SEAPorter™ HEK293 Cell lines are stable reporter cell lines that express intact and functional Toll-like Receptor as well as a secreted alkaline phosphatase (SEAP) reporter whose expression is under control of an NF- κB response element. These cell lines have been validated for use as reference markers for TLR expression by Flow Cytometry but are also ideally suited for applications which include:

- Examination of TLR activation pathways
- Screening for TLR and other inflammatory agonists and antagonists
- Scale-up for High-Throughput formats

Together these model systems provide key tools for further analysis of DC and T Cell Subsets, TLR agonists and antagonist immune/inflammatory signaling pathways and the immunoregulatory network which spans innate and adaptive immunity.

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Human Cutaneous Langerhans Cells are Stimulated by Alarmins in the Microenvironment to Initiate Th17 Responses

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Vaccination protocols that induce $CD4^+$ Th cells secreting IL-17 (Th17) have demonstrated enhanced efficacy by in part supporting neutrophil recruitment and maturation and the accumulation of memory Th1 cells. However, Th17 responses have also been linked to the induction of autoimmune diseases thus initiation of overly robust Th17 immunity may also be detrimental leading to severe tissue pathologies. Therefore, a better understanding of how Th17 responses are initiated in vivo is paramount in developing rational vaccines. Utilizing a model of human skin epidermal-dermal explants to collect skin migratory DCs (smiDCs) we have previously published that human cutaneous DCs are potent APCs capable of initiating co-existent Th17 and Th1 responses. This response was mediated exclusively by LCs, and not DDCs in an IL-6 and IL-15 dependent fashion, which is a different requirement from the described TGF-β and IL-21. In the present work we hypothesized that LCs are educated by alarmins specifically secreted into the microenvironment to drive their IL-15 expressing capacity. Indeed, we found that both ATP and HMGB1 are endogenous alarmins capable of stimulating smiDCs to initiate Th17 differentiation. Overall, our data demonstrate that human smiLCs are susceptible to microenvironmental alarmins and smiLCs induce Th17 responses by mechanisms different from those previously described. These findings highlight the need to target clinical treatments based on these environmental variations.

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Alpha(v) Integrin Expression by DCs is Required for Generation of Th17 Cells in Mice

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A distinct lineage of T helper cells, IL17-producing or Th17 T cells protect from bacterial and fungal infection, but also contribute to

inflammatory and autoimmune disorders such as multiple sclerosis. Th17 cells can be generated by exposure of naïve T cells to the immunoregulatory cytokine Transforming Growth Factor-beta (TGF-beta) in combination with proinflammatory cytokines. However less is known of how different antigen presenting cells contribute to Th17 generation and how this is regulated *in vivo*.

Here we show that differentiation of Th17 cells is critically dependent on alpha-v integrins, as conditional deletion of alpha-v from the immune system of mice results in loss of Th17 cells in the intestine and lymphoid tissues. Alpha-v integrins are similarly required for generation of pathological Th17 cells and alpha-v knockout mice are completely protected from experimental autoimmune encephalomyelitis (EAE). By both conditional gene targeting, and adoptive cell transfer, we have found that these effects are not due to intrinsic defects in T cells, but follow deletion of alpha-v in myeloid Dendritic cells (DCs) and macrophages. We show that DCs require alpha-v integrins to activate latent TGF- beta during T cell stimulation and thereby promote differentiation of Th17 cells. We further show that similar mechanisms act to generate adaptive Treg cells in the intestine, and we have identified specific subsets of DCs specialized for TGF- b activation.

These data demonstrate that activation of TGF- beta by alpha-v -expressing myeloid cells is a critical step in generation of both Th17 and peripheral Treg cells. We propose that expression of specific alpha-v integrins by DC and macrophage subsets is required for 'licensing' of T cell responses to TGF- beta in mucosal tissues, providing selective control over Th17 responses.

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Effects of EtOH exposure on bone marrow derived dendritic cell inflammatory response

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Dendritic cells (DCs) comprise a migratory group of bone marrow (BM) derived leukocytes able to uptake, process and present antigen. In this study, we evaluated the effects of ethanol (EtOH) exposure on DC effector functions. We differentiated DCs from BM obtained from 8-10 wk old C57BL/6 male mice. Total BM cells (2 x 10^6cells/mL) were cultured in the presence of GM-CSF (50ng/mL) and IL-4 (100U/mL) for 7 days. Following differentiation, DCs were harvested and pretreated for 3 hrs with increasing dosages of EtOH (50nM, 100nM, and 250nM) before being cultured in the presence of lipopolysaccharide (LPS) (1 □g/mL) for 24hrs. Following stimulation, supernatants were collected for cytokine measurement and cells were harvested for flow cytometry analysis. EtOH pretreatment (50nM, 100nM, and 250nM) resulted in a significant ($p<0.05$) dose dependent decreased secretion of pro-inflammatory cytokines IL-6 (~ 40%, 50% and 75%, respectively), IL-12(p40) (~ 0%, 20% and 40%, respectively), and IL-23 (~ 0%, 35% and 90%, respectively). Moreover, while untreated DC upregulated MHC II expression 2-fold following LPS stimulation, EtOH pretreatment blunted this response. Interestingly, EtOH pretreatment did not affect TLR4 expression. In summary, EtOH exposure, prior to antigenic stimulation, leads to suppression of pro-inflammatory cytokines and prevents up-regulation of MHC II expression in BM derived DC. Our study provides evidence to suggest that EtOH exposure may impair DC effector functions. (Supported by NIH R01AA015731 (MAC), T32AA013527, and the Dr. Ralph and Marian C. Falk Medical Research Trust).

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IL-17+ γδ T cells Promote Corneal Nerve Regeneration

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The contribution of acute inflammation to sensory nerve regeneration was investigated in the murine cornea using a model of corneal abrasion that removes the stratified epithelium and subbasalar nerve plexus. In this model, re-epithelialization and acute inflammation characterized by $CCR6^+$ $IL\text{-}17^+$ γδ T cell and neutrophil recruitment occur within the first day, but nerve regeneration progresses slowly. Morphometric analysis of regenerating nerve density was performed using a standard point counting grid with digital images of corneal whole mounts stained for tubulin βIII. Selective depletion experiments demonstrated that γδ T cells, IL-17, neutrophils and vascular endothelial growth factor (VEGF) were necessary for efficient corneal nerve regeneration following epithelial abrasion, resulting in >60% reductions in nerve density at 4 days after injury ($p<0.001$). γδ T cells were required for the recruitment and extravasation of neutrophils but appeared insufficient to directly promote nerve regrowth. The early rise of VEGF in the wounded cornea was significantly dependent on neutrophils, and spatial and temporal factors placed $VEGF^+$ neutrophils in proximity to very early regenerating neurites. Anti-VEGF marginally delayed re-epithelialization but markedly retarded nerve regeneration. These results indicate a beneficial role for the IL-17 and γδ T cell-dependent inflammatory response to corneal epithelial abrasion.

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Modulation of the Calcium Fluxes and Reactive Oxygen Species Response by Oleic Acid in Bovine Neutrophils

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Oleic acid is a nonesterified fatty acid (NEFA) released during lipomobilization in cows or following traumatic bone injury in humans. In human, it has been suggested that oleic acid could exert its effects via binding to G-protein coupled receptor GPR40, however its presence in bovine neutrophils is unknown. It has been described an effect of a blend of NEFA on bovine neutrophils such as a reduction on oxidative burst activity and viability. In this study, we assessed the effect of oleic acid on bovine neutrophils responses such as calcium mobilization, reactive oxygen species (ROS) production, adhesion molecules expression and apoptosis. Also, we examined the presence of GPR40.

Neutrophils were isolated from blood samples of healthy heifers. The calcium mobilization was assessed by spectrofluorimetry in Fluo 4-AM-loaded neutrophils. ROS production was assessed by luminescence assay and the superoxide and peroxide production by flow cytometry with the probe HE and DCFH-DA, respectively. Apoptosis was studied by flow cytometry with annexin V-IP staining and the CD11b and L-selectin expression by flow cytometry using specific antibodies. The presence of GPR40 was studied by real time-PCR.

Oleic acid induced intracellular calcium mobilization in bovine neutrophils. In a dose-dependent manner, oleic acid increased the superoxide release and reduced the peroxide production compared to the control. The pre-treatment of neutrophils with oleic acid (10 μM) and then with PAF or PMA showed a dose dependent synergistic effect in the superoxide release but a reduction of peroxide production compared to the stimuli. Oleic acid, in a dose dependent manner, increased the CD11b expression and reduced the L-selectin shedding. Only at 500 μM oleic acid we observed an increase in percentage of early apoptosis and death neutrophils. The mRNA of the bovine

ortholog of GPR40 in neutrophils bovine was detected. In conclusion, oleic acid increases intracellular calcium release, CD11b expression and superoxide production, nevertheless reduces the peroxide production and L-selectin shedding. Taken altogether, the results suggest a potential role of oleic acid on innate immune response during lipomobilization in cattle. Supported by grant from IFS (B/4720-1), Fondecyt 1090401 and DID-UACH.

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HUMAN C1Q DEFICIENCY IS LINKED TO AUTOIMMUNE DISORDERS

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C1q forms together with C1r and C1s the C1 macromolecule, the first component of the classical complement pathway. The formation of an antibody–antigen complex (immune complex; IC) is the principal way of activating the classical pathway of the complement system. C1q triggers the activation process when it docks onto antibodies within these immune complexes. In this way, C1q acts to bridge the innate and adaptive immune systems. Interaction of immune complexes with C1q induces a conformational change within the C1 complex, which results in activation of the classical pathway. C1q functions as recognition unit by binding to the heavy chain of IgG or IgM (Fc gamma and Fc micro) provided that the immunoglobulins are bound to their antigen. Furthermore, C1q can bind to apoptotic blebs, where it activates the classical complement pathway and mediates phagocytosis. As such, C1q promotes the clearance of apoptotic cells and subsequent exposure of auto-antigens, thereby preventing stimulation of the immune system. C1q is predominantly produced by macrophages but also by follicular dendritic cells, interdigitating cells and cells of the monocyte-macrophage lineage. C1q deficiency has a profound effect on host defense and clearance of immune complexes. Inherited C1q deficiency is also associated with the development of systemic lupus erythematosus (SLE). In the case of C1q deficiency, SLE is found in 90% of reported cases. C1q plays a role in the prevention of autoimmunity by facilitating the physiological clearance and processing of apoptotic debris. Absence of C1q may cause autoimmunity by impairment of the clearance of apoptotic cells. Low C1q levels are associated with proliferative glomerulonephritis (WHO class III and IV). Furthermore, C1q concentrations decrease prior to clinical manifestations of flares of the disease. Low C1q levels have also been shown to predict the histopathological outcome of lupus nephritis. A single nucleotide polymorphism in the *C1QA* gene results in decreased C1q serum levels and has been linked to photosensitive Lupus-specific skin disease, subacute cutaneous lupus erythematosus (SCLE). At Hycult Biotech we have developed a reliable ELISA to measure C1q as well as C1q-IC in serum, plasma and BALF.

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Propionate Induces Granules Release and Increases the Chemoattractant Response in Bovine Neutrophils

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Propionate is a short chain fatty acid (SCFA) produced under normal physiological conditions in the rumen of cattle. It is also involved in the inflammatory process and bovine neutrophil function via calcium release, reactive oxygen species and intracellular pH changes, however up today there is no evidence if propionate can induces granules release or exert a priming effect. Propionate has also been characterized as ligand for the G-protein coupled receptors, GPR41 and GPR43, thus it is possible that receptor dependent mechanisms may underlie some of these effects in bovine neutrophils. In this study, we assessed the effect of propionate on granules release, neutrophil responses such as calcium mobilization and the expression of GPR41 and GPR43 in bovine neutrophils. The release of the neutrophil enzymes matrix metalloproteinase 9 (MMP-9) and myeloperoxidase (MPO) was determinated by zymography, western blot and enzymatic assay.

Finally, to asses the expression of GPR41 and GPR43 mRNA we used RT-PCR. We have found that treatment of bovine neutrophil with 0.3 mM propionate induced MMP-9 release and at 30 mM increased the MPO release. We also found that propionate enhaced MMP-9 release induced by IL-8. We demostrated using Fluo-3AM loaded neutrophils that 30 mM propionate enhaced the calcium flux induced by 100 nM PAF. We assessed ERK1/2 and p38 MAPK phosphorylation by western blot and flow cytometry. We observed that propionate induces a rapid (5 minutes) phosphorylation of p38 MAPK and ERK 1/2. We also demostrated the expression of SCFA receptors GPR41 and GPR43 mRNA in bovine neutrophils. These results support the existence of a link between volatile fatty acid metabolism and the control of inflammatory and inmune response in bovines. This research was supported by Fondecyt 1090401, DID-UACH.

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TLR4 Plays a Role in Inflammatory Tolerance But Not in Impaired Bacterial Clearance in Mice after CLP

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Mice subjected to cecal ligation & puncture (CLP) have impaired ability to clear a subsequent bacterial challenge. Because cecal contents have a high LPS content, we conducted this study to determine if TLR4 played a role in the impaired innate immune response after abdominal contamination with cecal contents. Wild-type (WT) and TLR4-/- mice (C3H/ScNJ) received i.p. injections of heat-killed cecal contents isolated from mice and normalized for LPS (50ug per injection). 5 days later, the mice were challenged with live Ps. aeruginosa (1 x 10^8 cfu i.v.) and sacrificed 6 hours later. Plasma cytokines were measured; and spleens were aseptically excised, homogenized, and cultured overnight for quantification of Pseudomonas colony-forming units (cfus). WT mice subjected to abdominal contamination with heat-killed cecal contents had more Pseudomonas bacterial cfu's in spleen tissue (*5.2 x 10^4 vs 1.8 x 10^4); lower serum IFNg (*70 ± 15 vs 192 ± 43); lower serum TNFa (*82 ± 14 vs 166 ± 14); and higher serum IL-10 (*348 ± 43 vs 183 ± 42) than in WT control mice. TLR4-/- mice subjected to abdominal contamination also had higher numbers of Pseudomonas cfu's in spleen tissue than control TLR4-/- mice(*4.8 x 10^4 vs 1.9 x 10^4). However, TLR4-/- mice subjected to abdominal contamination did not differ in the serum IFNg, TNFa, or IL-10 response to the Pseudomonas challenge. TLR4 did not appear to play a role in the impairment of bacterial clearance after abdominal contamination with cecal contents. However, TLR4 did contribute to the suppressed proinflammatory and amplified anti-flammatory response to the Pseudomonas challenge in mice with previous abdominal contamination. (Supported by NIH-NIGMS).

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TLR4 differentially utilizes adaptor molecules MyD88 and TRIF to shape the CD4 T cell response to Hemagglutinin B from Porphyromonas gingivalis

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Porphyromonas gingivalis isa Gram-negative anaerobic bacterium and one of the main etiological agents contributing to adult chronic periodontitis. Immunization with recombinant Hemagglutinin B (rHagB), a virulence factor of the bacterium can render protection against bone loss following infection in an experimental

model.Our previous studies with dendritic cells (DC) showed that rHagB is a TLR4 agonist.Since DC are essential for the activation and differentiation of T cells, we investigated the role of TLR4 signaling in shaping the CD4 T cell responses following immunization of mice with rHagB.Immunization with rHagB resulted in the induction of antigen specific CD4 T cells that produced IFN-γ, IL-4, IL-5, IL-10, and IL-17, as well as a HagB specific IgG serum antibody response. Studies in $TLR4^{-/-}$ and $MyD88^{-/-}$ mice showed that there was an increase in the HagB specific type 2 CD4 T cell subsets, a decrease in type 1 subsets and that a higher ratio of serum HagB specific IgG_1/IgG_2 antibodies was seen when compared to wild type (WT) mice.Furthermore, an increase in GATA-3 and Foxp3 expression and a decrease in CD4 T cell activation were observed in $TLR4^{-/-}$ and $MyD88^{-/-}$ mice.Moreover, $TLR4^{-/-}$ CD4 but not $MyD88^{-/-}$ CD4 T cells showed an increased IL-2/Stat5 signaling.TRIFdeficiency resulted in increased IFN-γ and IL-17 production by HagB specific CD4 T cells and this was accompanied by an increased T-bet and ROR-□t expression and CD4 T cell activation.These studies show for the first time the importance of TLR4 signaling in the CD4 T cell response to the *P. gingivalis* antigen HagB, using the downstream MyD88 and TRIF adaptor molecules. The information gained is most relevant for the future design of therapeutic strategies against periodontal diseases. (Supported by grants DE 14215 from NIH and T32 AI-007051 from NIAID, NIH.)

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Extraction of mRNA from coagulated horse blood and analysis of inflammation-related cytokine responses to coagulation.

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Coagulated blood is a rich source of mRNA that allows the study of the regulation of expression of cytokine and other genes. However, while several methods are available for isolation of RNA from whole blood and tissues, protocols for purification of mRNA from clotted blood are not generally available. Here, a protocol for RNA extraction from highly clotted blood was optimized and the regulation of a number of cytokine genes compared to stabilized blood was studied. Whole blood samples from 10 clinically healthy horses were incubated for 24 hours at 37°C and RNA was extracted from the peripheral blood mononuclear cells present in the blood clot, homogenizing the clot by rotating knife homogenization (GentleMACS, Miltenyi Biotec) in the presence of QIAzol extraction buffer (Qiagen). The RNA extracted yielded high concentrations of total RNA (50-265 ng/µl) and quality measures (RIN=8.5-9.2), comparable with that purified by standard methods from stabilized blood. Cytokine mRNA expression was assessed by reverse transcribed quantitative real time PCR and it was found that 24-hour clotting led to a significant increase in the concentrations of mRNA of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-1-receptor antagonist (IL-1ra), interleukin-15 (IL-15), and interleukin-8 (IL-8). These findings that a coagulation-induced inflammation-related cytokine response takes place in whole blood upon clotting. The extraction method provides reproducible and reliable results allowing the recovery of quantifiable high-quality RNA for molecular expression analysis.

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Differential responses to inflammatory stimulation in Sprague Dawley rats obtained from different vendors

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Previous studies suggest that animals obtained from different vendors may vary in their immune response to inflammatory challenge. We investigated whether Sprague Dawley (SD) rats obtain from Harlan Laboratories (Kent, WA USA) and Charles River Laboratories

International (Saint-Constant, QC, Canada) show differential susceptibility to and course of adjuvant-induced arthritis (AA). The AA model results in chronic inflammatory stress, simulating the human experience of rheumatoid arthritis, a T-cell mediated autoimmune disease. Due to the high degree of interconnectivity between the hypothalamic-pituitary-adrenal (HPA) axis and the immune system, we hypothesized that SD rats from the two vendors would differ at the level of both the neuroendocrine and immune system, following AA induction. Functional immune status was assessed in adulthood following injection of Complete Freund's Adjuvant (CFA) to initiate AA. High (1.2 mg) and low (0.3 mg) doses of CFA were tested in all animals, and two additional doses (0.6 mg in Charles River and 0.2 mg in Harlan) were selected based on the initial responses to the high and low CFA doses. Harlan rats exhibited a greater incidence and greater severity of AA, compared to Charles River animals, as demonstrated by a greater attenuation of weight gain, higher clinical scores and increased paw swelling at both the high and low CFA doses. In addition, Harlan animals exhibited greater corticosterone (CORT) responses to inflammatory challenge, with CORT levels increasing as AA severity increased. Overall, our findings indicate that there are vendor differences at the level of the neuroendocrine-immune response to inflammation. Future analyses will examine levels of pro- and anti-inflammatory cytokines in plasma as well as local cytokine levels in the affected hind paw joints and in the brain in order to further investigate the mechanism underlying this altered immune function in SD rats from the two vendors.

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Delphinidin induces calcium mobilization and the production of IL-2 through of the store-operated calcium entry in T cells.

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Delphinidin is an anthocianidin frequently consumed in the diets which however been suggested to exert a number of beneficial actions on human health. This molecule has demonstrated multiple biological properties including antioxidant and anti-inflammatory activities. Their properties have been studied in numerous cell types but is unknown their effect on T cell biology. In the present study, we determined that delphinidin increase the production and expression of interleukin-2 (IL-2) in T cells via "Store-operated calcium Ca^{+2} entry" (SOCE). First, by using BAPTA and EGTA, we observed that delphinidin increased cytosolic-free Ca^{2+} by releasing Ca^{+2} from intracellular store and by increasing Ca^{+2} entry in a dose-dependent manner in Jurkat cells, by spectrofluorimetrics experiments and images analyses. Next, we tested the efficacy of several putative SOCE inhibitors such as 2-aminoethoxydiphenyl borate (2-APB), N-(4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5 carboxamide (BTP-2) and gadolinium (Gd^{3+}) on Ca^{+2} entry induced by delphinidin. BTP2, 2-APB and Gd^{+3} reduced significantly the calcium entry stimulated by delphinidin in a dose-dependent manner.
To asses if SOCE contributes to the IL-2 production induced by delphinidin, we treated the Jurkat cells and lymphocytes with a putative SOCE inhibitor such as BTP2. BTP2 significantly decrease the IL-2 production induced by delphinidin at concentration of 1 µM, 5 µM and 10 µM in both cell types.

Moreover, we observed that the expression of the IL-2 gene in Jurkat cells pre-incubated with BTP-2 was significantly decreased as compared to cells stimulated with delphinidin . With these results, we concluded that delphinidin can exert immunostimulant effect via SOCE in T cells. This research was supported by CONICYT, MECESUP UCO 0606, DID U.A.Ch and Consorcio de Tecnología e Innovación para la Salud (CTI-Salud S.A.) PBCT-6.

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2-Aminoethoxydiphenyl borate (2-APB) reduces respiratory burst, MMP-9 release and CD11b expression, and increases L-selectin shedding in bovine neutrophils

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This study describes the effect of 2-Aminoethoxydiphenyl borate (2-APB), a putative store-operated calcium (Ca^{2+}) entry (SOCE) inhibitor, on reactive oxygen species (ROS) production, matrix metalloproteinase 9 (MMP-9) release, CD11b and L-selectin (CD62L) expression, size changes and apoptosis in bovine neutrophils stimulated with platelet-activating factor (PAF) and/or zymosan. Neutrophils were obtained from whole blood and purified by a hypertonic lysis step. The cells were washed in HBSS and incubated for 15 min with 2-APB in a range of concentrations from 1µ M to 100µ M. Afterwards the neutrophils were stimulated with PAF 10^{-7}M or zymosan 1 mg ml^{-1} for the desired time. The ROS production was measured using the luminol method and the probe DCFH-DA by luminometry and spectrofluorometry respectively. MMP-9 secretion and activity was determined using zymography and the results were confirmed by inmunoblot using anti-MMP-9 antibodies. CD11b expression and L-selecting shedding were analyzed by flow citometry. We observed that doses ≥1 µ M 2-APB significantly reduced ROS production induced by PAF whereas concentrations ≥50 □Mreduced the ROS production induced by zymosan, 50 □Mand 100 □M2-APB reduced MMP-9 release induced by PAF. Moreover, concentrations ≥10 □M2-APB reduced CD11b expression and increased L-selectin shedding. PAF induced size changes in neutrophils, and this effect was inhibited by 2-APB. Finally, we observed that 2-APB did not affect the viability of the cells. We concluded that 2-APB at concentrations that inhibit SOCE responses was able to inhibit ROS and MMP-9 release and CD11b expression, and increase L-selectin shedding, suggesting that the Ca^{2+} channel involved in SOCE is key in innate immune response and a potential target for the development of new anti-inflammatory drugs in cattle.

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A Role for Delta-PKC (δ-PKC) in the Regulation of Neutrophil Recruitment to the Lung

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Sepsis and systemic inflammation activate a cascade of proinflammatory events that can lead to excessive neutrophil infiltration and initiation of lung tissue damage. Cytokines such as IL-1/TNF activate pulmonary endothelium and enhance neutrophil transmigration. We identified δ-PKC as a regulator of cytokine-mediated pro-inflammatory signaling. Thus, inhibition of δ-PKC may control cytokine-elicited neutrophil migration into the lung. In vitro, cytokine (IL-1, 10U/ml) treatment of pulmonary microvascular endothelial cells (PMVEC) triggered activation of δ-PKC and increased expression of the adhesion molecules ICAM-1 and VCAM-1. Pretreatment of PMVEC with a δ-PKC-TAT inhibitory peptide blocked cytokine-mediated upregulation of ICAM-1 and VCAM-1 expression. In contrast, PECAM-1 expression was both cytokine and δ-PKC independent. IL-1 treatment of PMVEC monolayers enhanced neutrophil binding in a δ-PKC-dependent manner. IL-1 treatment also enhanced neutrophil transmigration through PMVEC grown on transwells as compared to unstimulated PMVEC. Inhibition of δ-PKC

decreased neutrophil transmigration by >50%. Neutrophil migration through cytokine-activated endothelium is β2-integrin-dependent. Unstimulated neutrophils have little expression of the β2-integrin, CD11b. Transmigration through IL-1-activated PMVEC monolayers significantly increased neutrophil CD11b expression which was δ-PKC dependent. Using a rat model of sepsis and ARDS (cecal ligation and double puncture (2CLP)), we examined the role of δ-PKC in neutrophil migration into the lung. Following 2CLP surgery, rats received either intra-tracheal injection of PBS or the δ-PKC-TAT peptide (200ug/kg). A control group underwent sham surgery. At 24hrs post surgery, neutrophil influx was determined by fluorescent staining of myeloperoxidase (MPO)-positive cells in lung tissue. Septic animals treated with vehicle (PBS) had enhanced numbers of MPO-positive cells infiltrating the lungs as compared to sham-operated controls. In contrast, septic animals which received the δ-PKC-TAT peptide had significantly reduced numbers of neutrophils in the lung. These results indicate an important role for δ-PKC controlling neutrophil:endothelial interactions, neutrophil transendothelial migration and recruitment to the lung. (Supported by NIH GM64552).

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Crosstalk between estrogen/estrogen receptor and IL-6/Jak/Stat3 signal in pathogenesis of sodium arsenite-induced renal injury

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We examined the gender difference of susceptibility to sodium arenite (NaAs)-induced renal injury in mice. BALB/c mice of both genders were intraperitoneally injected with 12.5 mg/kg of NaAs. Female mice exhibited exaggerated elevation of serum blood urea nitrogen and creatinine levels, compared with male ones. Consistently, h istopathological alterations of the kidneys such as severe hemorrhages, acute tubular necrosis, cast formation, and disappearance of PAS-positive brush borders were more evident in fem ale mice, later than 6 hrs. Ovariectomy significantly attenuated NaAs-induced renal injury in female like male mice. On the other hand, treatment of male mice with fultamide, an inhibitor of androgen receptor, failed to exaggerate renal injury. These facts suggest that estrogen/estrogen receptor signal is involved in this gender difference. Autophagy and ERK activation were induced to a higher extent in female mice challenged with NaAs. We demonstrated that IL-6/Stat3 signal pathway played protective roles in NaAs-induced renal injury by the suppression of tubular cell autophagy through inhibition of ERK activation. Therefore, it is likely that IL-6/Jak/Stat3 signaling is involved in the gender difference of susceptibility to NaAs nephrotoxicity. Moreover, in IL-6-deficient mice, male mice showed higher susceptibility to NaAs with higher extents of autophagy, compared with female ones. Consistently, Stat3 phosphorylation was significantly reduced in the kidney of female mice administrated with NaAs, compared with male mice. Taken together, estrogen/estrogen receptor signaling enhances ERK activation by inhibition of IL-6/Stat3 signaling pathway in NaAs-induced renal injury, resulting in higher susceptibility to NaAs nephrotoxicity in female mice.

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Individual cell tracking in a transgenic zebrafish inflammation model reveals the fates of inflammatory neutrophils during inflammation resolution

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Removal of inflammatory neutrophils from sites of inflammation can occur by a number of routes; into exudates, by apoptosis followed by macrophage clearance and by retrograde chemotaxis. The relative contribution of these disposal mechanisms in vivo has been hard to define, and the lifespan of an *in vivo* tissue neutrophil has been hard to directly measure. We have generated transgenic zebrafish expressing fluorescent proteins (GFP or photo-convertible Kaede) in neutrophils, and mCherry in macrophages.

Objective: To label individual inflammatory neutrophils and track their fate during inflammation resolution *in vivo*.

Method: Individual neutrophils were marked by photoconverting the Kaede protein using 405nm laser light restricted to the individual cell profiles. Known numbers of neutrophils were photoconverted and visualised over 48 hours. In subsequent experiments, an inflammatory reaction was induced by sterile tail transection of transparent zebrafish larvae. Kaede labelled neutrophils are recruited to the site of injury where they can be photoconverted and followed using timelapse videomicroscopy. Finally, u sing macrophage and neutrophil-specific double transgenic zebrafish, we have studied cell specific behaviours and neutrophil-macrophage interactions during inflammation.

Results: By counting the number of remaining photoconverted neutrophils over time, the half-life of a neutrophil was calculated. Our data suggest the lifespan of a zebrafish neutrophil in the tissues is 117.7 (CI 95.67-157.8) hours, a figure comparable to that inferred for human tissue neutrophils.

Timelapse videos reveal a population of neutrophils that migrate away from the site of injury, undergoing retrograde chemotaxis. Whilst neutrophils can migrate away from the site of injury, they are not completely free to do so. The apparent restriction on their behaviour may be due to the presence of a persisting chemical gradient or may reflect an intrinsic feature of neutrophil behaviour.

Neutrophils and macrophages exhibit different migration patterns, with neutrophil numbers peaking at 6 hours post injury. In contrast, macrophage numbers peak at 10 hours post injury, and persist while neutrophil numbers spontaneously fall. Neutrophil apoptosis and macrophage uptake is readily visualised during inflammation resolution.

Conclusions: These data demonstrate the power of this model to inform our understanding of phagocyte behaviour and interaction in vivo.

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SHIP deficiency is required for alternative activation of macrophages and is a potential target for intervention in inflammatory bowel disease

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Macrophages (mΦs) play a key role in the innate immune system by initiating and directing its response. Classically activated (M1) mΦs promote inflammation and then are skewed to alternatively activated (M2) mΦs that promote the healing response. The src homology 2 domain-containing inositol-5'-phosphatase (SHIP) is a hematopoietic specific negative regulator of the PI3K pathway and mΦs from SHIP deficient mice are profoundly M2 skewed. Based on this observation, we asked if SHIP is required for M2 skewing. We have found that reduced SHIP levels are required for M2 skewing. PI3Kp110delta and STAT6 are required for SHIP degradation and expression of alternative activation markers. Importantly, by manipulating SHIP expression in a mΦ cell line

we can affect M1 or M2 skewing. M2 mΦs have been shown to be protective in animal models of intestinal inflammation. Based on this observation, we asked whether genetic models of polarized mΦ phenotype were affected during dextran sodium sulfate (DSS)-induced colitis. SHIP-/- mice (M2 skewed) were protected during DSS-induced colitis while PI3Kdelta-/- mice (M1 skewed) showed exacerbated inflammation. Current studies focus on using chlodronate liposomes to deplete mΦs during the DSS model and to reconstitute mΦs with *in vitro* derived M1 or M2 mΦs to see if we can manipulate the severity of disease. Skewing mΦs toward an alternatively activated phenotype is a promising strategy for treating inflammatory bowel disease so it is important to identify the critical switches required for mΦ skewing. This research is supported by the Crohns's and Colitis Foundation of Canada

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Polyisoprenyl diphosphate phosphatase1 regulates leukocyte functional responses to soluble stimuli.

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Presqualene diphosphate (PSDP) is an intracellular signaling molecule in human neutrophils (PMNs). Activation of PMNs results in rapid, within seconds, and transient conversion of PSDP to presqualene monophosphate (PSMP), while the addition of exogenous PSDP, but not PSMP, blocks PMN activation in response to soluble stimuli. Polyisoprenyl diphosphate phosphatase 1 (PDP1) is a regulated enzyme that converts cellular PSDP to PSMP. Here, we present evidence that PDP1 activation is linked to PMN functional responses and is regulated by select protein kinase C (PKC) isoforms. HL60 cells were chosen for initial study of PDP1's role in cellular functional responses because, under select conditions, HL60 cells can be differentiated to cells with many characteristics of human PMNs. HL60 cells that stably express PDP1 siRNA had a 22% reduction in PDP1 mRNA levels compared to mock transfected cells. This subtle change in expression in differentiated HL60 cells led to dramatic decreases in leukotriene B_4 mediated adherence to fibronectinin and production of superoxide anions in response to soluble stimuli. Lipoxin A_4 (LXA_4) isa potent counter-regulator lipid mediator that blocks the conversion of PSDP to PSMP in human PMN. LXA_4 decreased *f*MLP initiated O_2- production in PDP1 siRNA cells in a manner consistent with signaling through PDP1. The rapid conversion of PSDP to PSMP in response to agonist suggests that PDP1 activity is regulated by post-translational modification. Amino acid analysis uncovered several potential PKC phosphorylation sites in PDP1. Experiments demonstrate that rhPDP1 is phosphorylated by PKC βII and PKC ζ *in vitro* and inhibitors specific for the aforemeantioned PKCs block *f*MLP-mediated PSDP to PSMP remodeling in human PMN. In conclusion, our data provide evidence that PDP1 is a regulated PSDP phosphatase that may serve as a pivotal checkpoint in the regulation of PMN functional responses.

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Arginase Activity is a Therapeutic Target in Treating Fibrosis

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Crohn's Disease is the result of an inappropriate immune response in the gut leading to intestinal inflammation. Intestinal fibrosis is a serious complication in patients with Crohn's Disease which can result in stricture formation often requiring surgery. Fibrosis is the result of a build up of collagenous scar tissue from a robust and sometimes overzealous healing response. Src homology 2 domain-containing inositol 5´-phosphatase (SHIP) knockout (SHIP-/-) mice are a genetic model of profoundly alternatively activated macrophages that express high levels of arginase I. We have found that SHIP-/- mice spontaneously develop intestinal inflammation that is evident as early as 4 weeks of age and that shares features with Crohn's Disease such as discontinuous, transmural inflammation and granulomatous structures in the submucosa and gut-associated lymphoid tissue. In the SHIP-/- mice, inflammation was restricted to the distal ileum, while colons and ceca appeared normal. The major components of the inflammatory infiltrates and lymphoid aggregates were Gr-1+ neutrophils with clusters of CD3+ T cells . Cytokine profiling of tissue explants from sites of active ileal inflammation revealed elevated TNFα and IL-10. Immunohistochemistry for arginase I expression correlated with elevated arginase activity in the SHIP -/- mouse ileum relative to wild type littermates. Purified SHIP-/- ileal macrophages expressed high levels of the M2 markers arginase I and Ym1, which correlated with F4/80+ argI+ macrophage staining in tissue sections. Masson's trichrome staining for fibrosis and Sircol assays for soluble collagen revealed increased collagen deposition in SHIP-/- mice compared to wild type littermates. Daily administration of 0.2% S-2 boronoethyl-cysteine (BEC) for 14 days inhibited collagen deposition in the distal ileum however inflammatory infiltrates were still present. Our results demonstrate that in hibition of arginase weakens intestinal fibrosis in a new murine model of intestinal inflammation. This suggests that arginase I is a potential therapeutic target in treating intestinal fibrosis. Funding was provided by the Canadian Association of Gastroenterology, the Crohn's and Colitis Foundation of Canada and the Canadian Institutes of Health Research.

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Resolvin D1 Modulates Adipose Tissue Macrophage Accumulation and Restores Insulin Sensitivity in Obese Diabetic Mice

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Obesity is associated with the accumulation of classically activated macrophages in adipose tissue, which contribute to chronic low-grade inflammation and systemic insulin resistance. Resolvins are endogenous leukocyte-derived lipid mediators generated from omega-3 fatty acids that actively promote the resolution of acute inflammation, in part via direct actions on macrophages. We sought to determine if resolvin D1 (RvD1) modulates macrophage accumulation in obesity-induced diabetes. In leptin-receptor deficient (db/db) mice, RvD1 (2μg/kg per day) improved glucose tolerance, decreased fasting blood glucose levels and increased insulin-stimulated Akt-1 phosphorylation in adipose tissue. Importantly, phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and circulating levels of adiponectin were also increased in db/db mice given RvD1. In RvD1-treated animals, the formation of crown-like structures rich in $F4/80^+$ $CD11c^+$ macrophages was decreased in adipose tissue. Treatment with RvD1 increased the percentage of $F4/80^+$ macrophages expressing the galactose-type C-type lectin 1 (Mgl-1/CD301), a marker of alternatively activated macrophages. Thus, RvD1 decreases inflammatory adipose-tissue macrophage accumulation in obesity-induced diabetes and restores insulin sensitivity.

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Profound Reduction of Invariant Natural Killer T Cells in the Peripheral Blood of a Patient with IRAK-4 Deficiency

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A female patient with IRAK-4 deficiency (Picard *et al.*, *Science* 2003) associated with life-threatening pneumonia and frequent infections caused by invasive pyogenic bacterial pathogens including *S. pneumoniae* and *S. aureus* in the absence of fevers and defective production of IL-12, TNF-α , and IFN-γ upon stimulation of her PBMC with LPS or fixed *S. aureus* Cowan strain 1was first reported by us (Haraguchi *et al.*, *PNAS* 1998). The patient was unable to maintain protective titer of antibodies to polysaccharide or protein antigens and to a neoantigen bacteriophage (Day *et al.*, *J. Pediatr.* 2004). In the present study, we show that the patient exhibits a profound absence of invariant NKT (iNKT) cells (0.00%) in the peripheral blood, as assessed by flow cytometry using anti-human iNKT mAb 6B11, anti-TCR Vβ 11 mAb, and anti-CD3 mAb. By contrast, the frequency of iNKT cells in nine healthy controls ranged from 0.05 to 0.39% (mean = 0.18%). *Ex vivo* expansion of iNKT cells for 7 days in the presence of iNKT cell-specific agonist α -GalCer showed that <0.005% iNKT cells from the patient were amplified when compared with cells from three healthy controls (32.70, 5.77, and 2.75%, respectively). In conclusion, we show that our patient with IRAK-4 deficiency has an additional defect in the innate immune system. This "experiment of nature" will help us to understand human iNKT-cell development and homeostasis as well as the role of human iNKT cells in immunological disorders and host defense against pathogens. Dedicated to the memory of Robert A. Good. Supported in part by the Eleanor Naylor Dana Charitable Trust and the Talecris Biotherapeutic Inc. Educational grant (NKD).

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Pgp activity in γδ cells segregates with CD27 expression and location in the body

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P-glycoprotein (Pgp) is a member of the ABC family of transporter proteins which are characterized by their ability to pump molecules across membranes in an ATP-dependent manner. Although Pgp was first identified for its ability to confer resistance to chemotherapeutic agents in tumor cells, it has also been described in normal cells of the immune system. Our work primarily focuses on γδ T cells that complement and regulate the activities of αβ T cells, particularly in tissues. We have recently described functional subsets of γδ cells based on CD27 expression. $\gamma\delta^{27+}$ cells secrete IFNγ, while $\gamma\delta^{27-}$ cells are capable of producing IL-17. This study investigates the role of Pgp in γδ cells with specific reference to these recently-identified CD27-defined subsets. *Results:* Up to 40% of mice intraepithelial lymphocytes (IELs) from the small intestine are TCR $\gamma\delta^{+}$. Of these, virtually all displayed Pgp activity. Indeed, Pgp activity was generally higher in TCR $\gamma\delta^{+}$ than TCR $\alpha\beta^{+}$ IELs. In the thymus, Pgp activity was observed in only ~2% of $\gamma\delta^{27+}$ cells but not at all in $\gamma\delta^{27-}$ cells. By contrast, in peripheral lymph nodes, mesenteric lymph nodes and Peyer's Patches, 40-60% of $\gamma\delta^{27+}$ cells were positive for Pgp activity, although their $\gamma\delta^{27-}$ counterparts remained

largely negative. Moreover, $CD27^{+}Pgp^{(+)}$ produced more IFNγ than $CD27^{+}Pgp^{(-)}$ but there was no difference on IL-17 expression, and $CD27^{+}Pgp^{(+)}$ does not express CCR7 while $CD27^{+}Pgp^{(-)}$ do express. With regard to human cells, we observed that more than 90% of human V γ 9^{+} γδ cells have Pgp activity. In a malaria mouse model we did not observe any alteration in Pgp activity in γδ T cells. *Conclusion :* This study demonstrates that subsets of γδ cells display different levels of Pgp activity depending on their location in the body and their expression of the newly identified functional marker CD27. As Pgp activity may play a role in cytokine release, cytotoxicity and protection from harmful toxins, it confirms our hypothesis that $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ cells have very different roles in immune responses and provides insight into the mechanism by which γδ cells cope with diverse body locations. *Financial support :* CNPq, Capes, FAPERJ.

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Reduced TIMP-2 In Hypoxia Enhances Angiogenesis

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Hypoxia, which characterizes ischemia, trauma, inflammation and solid tumors, recruits monocytes, immobilizes them and alters their function, leading to an anti-inflammatory and pro-angiogenic phenotype. Monocyte extravasation from the circulation and their migration in tissues are partially mediated by the balance between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). The mechanisms evoked by hypoxia that regulate monocyte migration and activation are not entirely clear. Specifically, the effect of hypoxia on TIMPs in these cells was hardly investigated. We show that hypoxia reduces TIMP-2 secretion from human primary monocytes and from the monocytic-like cell lines U937 and THP-1 by 3-4 fold ($p<0.01$), by inhibiting TIMP-2 transcription through mechanisms which involve the transcription factor SP-1. Hypoxia also lowers TIMP-2 protein secretion from human endothelial cells (by 2-fold, $p<0.05$). TIMP-2 levels do not influence the reduced migration of THP-1 cells in hypoxia, however low TIMP-2 levels enhance endothelial cell migration/proliferation, their ability to form tube-like structures *in vitro* and the appearance of mature blood vessels in a Matrigel plug assay *in vivo*. Thus, we conclude that reduced TIMP-2 levels secreted from both hypoxic monocytes and endothelial cells are pro-angiogenic.

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Roles for Lymphocytes and Non-hematopoietic Cells in the Pathogenesis of Chronic Skin Inflammation in IκB-ζ-deficient Mice

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I κB-ζ is a nuclear I κB protein that is induced via stimulation of Toll-like receptors or the interleukin (IL)-1 receptor. The induced I κB-ζ associates with NF- κB in the nucleus and regulates its target genes. In macrophages, I κB-ζ is essential for induction of the secondary response genes such as IL-6 and IL-12 in response to stimulation by the Toll-like receptor ligands. I κB-ζ knockout (KO) mice spontaneously develop periocular and perioral skin inflammation with heavy $CD4^{+}$ cell and $B220^{+}$ cell infiltration into the epidermis at 3 to 8 weeks old under the specific pathogen free conditions. Although critical roles for tumor necrosis factor (TNF)- α have been reported in skin inflammation of IKK- □6□ □KO mice, I κB-ζ /TNF- α double KO mice exhibited similar inflammation, underscoring clear differences in the pathogenesis of the inflammations. Up-regulation of mRNAs for various proinflammatory cytokines and chemokines was observed in the inflamed skin of I κB-ζ KO mice. Interestingly, up-regulation of mRNAs for CXCL9 and 10 was found in the periocular skin of the 2-week-old I κB-ζ KO mice that did not

exhibit apparent inflammation. In the I κB-ζ / RAG2 double KO mice lacking mature lymphocytes, the inflammation was dramatically ameliorated, whereas CXCL9 and 10 were up-regulated, indicating critical roles for lymphocytes in the development of the inflammation. On the other hand, adoptive transfer of splenocytes either from the wild-type or I κB-ζ KO mice to I I κB-ζ / RAG2 double KO mice induced severe skin lesion. Furthermore, bone marrow chimera with I κB-ζ -deficient hematopoietic cells did not exhibit the skin lesion, demonstrating that I κB-ζ deficiency in lymphocytes is not sufficient for the onset of the inflammation. Therefore, expression of I κB-ζ in non-hematopoietic cells is important in skin homeostasis.

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Constitutive expression of CD25 on the surface of neonatal (invariant) Natural Killer T cells primes them to proliferate with lower antigenic stimulation

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Invariant Natural Killer T (iNKT) cells in neonates are known to constitutively express the IL-2 receptor alpha chain (CD25). We explored the functional consequence of the expression of CD25 on the surface of neonatal iNKT cells by comparing their functional properties to other common CD25-expressing T cell subsets. Unlike CD25-expressing activated T cells, iNKT cells seem to express high levels of Kruppel-Like Factor-2, a transcription factor highly expressed in quiescent T cells. Furthermore, neonatal iNKT cells differ from CD25+CD4+ T regulatory cells as they do not suppress conventional T cell proliferation. Upon stimulation with CD1d-restricted antigens, neonatal iNKT cells display a dramatically lower proliferation threshold compared to adult iNKT cells. Further experiments using a competitive blocking IL-2 antibody suggest that the markedly reduced proliferation threshold of neonatal iNKT cells is, at least in part, due to the constitutive expression of CD25. The functional consequence of CD25 expression on neonatal iNKT cells appears to be distinct from that of activated T cells, in that the former do not proliferate when exposed to IL-2 in the absence of T cell receptor (TCR)/CD28 co-stimulation. In light of the limited structural diversity of iNKT cells' TCR, we speculate that neonatal iNKT cells' unique and distinct phenotype plays a role in the maintenance of a diverse repertoire with repeated low avidity antigenic challenge early in life.

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New TLR4-active glycolipids selectively targeting CD14 and MD-2 co-receptors

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Bacterial lipopolysaccharide (LPS) is recognized by the innate immune system of vertebrates via an elaborate mechanism involving the membrane Toll-like receptor 4 (TLR4) and the co-receptors LBP, CD-14 and MD-2. Small molecules selectively targeting these receptors have great potential as new therapeutic agents and are unique molecular tools to study the process of LPS sensing and signaling by means of a "chemical genetic" approach. In this communication we present the synthesis and the biological characterization of new small molecules, derived from natural sugars, that are active on the TLR4-mediated LPS signaling. Cationic glycolipids (CGL) have been developed by our group, that inhibit lipopolysaccharide (LPS) and lipid A-induced cytokine production in innate immunity cells[1, 2]. CGL are also active in inhibiting septic shock in vivo and other syndromes caused by TLR4 activation, such as chronic pain and neuropathic pain [3]. Biochemical in vitro studies with purified TLR4, MD-2 and CD14 receptors indicated that these molecules inhibit the TLR4 pathway by selectively antagonizing the endotoxin binding to CD14 [4]. The discovery of another class of synthetic glycolipids with a disaccharide scaffold and negatively charged sulfate or phosphate groups, active as mild agonists of TLR4, will be also described. These molecules selectively target the MD-2 receptor and are promising leads for the development of nontoxic vaccine adjuvant and immunotherapeutics.
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Regulation of proteinase-activated receptor-2 by Fc-gamma receptors in human neutrophils: Elucidation of a major signaling pathway for TNF-alpha generation

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Proteinase-activated receptors (PAR-1 to PAR-4) regulate cell survival and proliferation as well as lipid mediator, cytokine and neuropeptide release. They can also induce all of the symptoms of inflammation. Understanding of the direct impact of PARs on leucocyte activities remains incomplete.
Objectives. To decipher the events that control PAR expression in human neutrophils and to identify PAR-dependent cellular responses.
Methods. Gene expression in blood neutrophils from healthy donors was measured by real-time PCR; cell-surface expression was monitored using FACS; involvement of signal transduction pathways was assessed with specific inhibitors; release of soluble factors was quantified using ELISA.
Results. Expression of PAR-2 and PAR-3 mRNA was constitutive. Stimulation of neutrophils with opsonized bacteria (Bop) specifically up-regulated cell surface expression of PAR-2, independently of transcription or de novo protein synthesis. Primary granules were identified as the main source of pre-formed PAR-2 readily mobilized at the surface upon fusion with the plasma membrane. Cellular response to PAR-2 activation, measured as changes in intracellular calcium concentration, was enhanced in PAR-2 up-regulated cells. Increases of cell-surface PAR-2 and cell

responsiveness were dependent specifically on the engagement of immunoglobulin (Ig)-binding (Fc-gamma) receptors. PAR-2 activation induced the generation and release of tumor necrosis factor (TNF)-alpha. Among the various conditions tested, PAR-2 activation was the most potent stimulator of TNF-alpha secretion.
Conclusions. Contact between neutrophils and IgG-coated particles up-regulates PAR-2 surface expression; PAR-2 activation appears to be one of the strongest signals for TNF-alpha generation by neutrophils.
Support. This work received funding from the Canadian Institutes of Health Research (CIHR, IMH-102820) and the U.S. National Institutes of Health (R01 AR052614). MP is a senior investigator with support from the *Fonds de la Recherche en Santé du Québec* (FRSQ).

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IL-18 induced immune cell apoptosis in female mice but not in male mice during lethal endotoxemia

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Introduction Delayed-granulocyte-apoptosis is implicated in persistent inflammation, while induced bone-marrow-cell-apoptosis leads to immunosuppression. Although, it is known that female gender develop sepsis less frequently than male. Serum IL-18 levels correspond to severity of systemic inflammation.
Objective The purpose of this study was to elucidate gender differences in response to LPS stimulation and the effects of IL-18 on granulocyte dynamics and its apoptosis.
Material and Methods Male and female wild-type (WT) and IL-18 knockout (KO) mice were injected intraperitoneally with LPS. Bone marrow (BM) cells, peripheral blood and peritoneal cells were then collected at 0, 18 and 24 hrs after injection. Apoptosis was assessed by annexin V staining and each cell population was defined by staining with granulocyte-specific Gr-1, B-lymphocyte specific B220 and macrophage specific F4/80 antibodies using three-color-flow cytometry.
Results The survival rate was approximately 40, 60, 80 and 80% in WT male, KO male, WT female and KO female mice, respectively. The numbers of BM cells were decreased in both WT and KO mice irrespective of gender. The apoptosis of BM cells (myeloblast, immature cell and B-lymphoblast) and peripheral cells (neutrophils and lymphocytes) in WT female mice were significantly higher than in WT male mice. The number of neutrophils in the peritoneal cavity in WT female mice was significantly lower than in WT male mice, and the apoptosis of peritoneal neutrophils, macrophages and lymphocytes in WT female mice were significantly higher than in WT male mice. Such apoptotic tendency in WT female mice was not seen in KO female mice while there was no difference between WT and KO mice in male gender.
Conclusion IL-18 seems to play a pivotal role in apoptosis induction of lymphocyte and neutrophils in BM, blood and peritoneal cavity in female but not in male mice.

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A Novel Isolation Method for Macrophage-like Cells from Mixed Primary Cultures of Adult Rat Liver Cells

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We report a simple and efficient method to obtain macrophage-like cells from the mixed primary cultures of adult rat liver cells. A parenchymal hepatocyte enriched fraction was prepared from adult rat livers and seeded into culture flasks. After 7 to 10 days of culture, when most hepatocytes were degenerated or transformed into fibroblastic cells, macrophage-like cells vigorously proliferated on the cell sheet. By shaking the flasks, macrophage-like cells were readily detached. Subsequent transfer and incubation in plastic dishes resulted in quick and selective adhesion of macrophage-like cells, while other contaminating cells remained suspended in the medium. After rinsing with saline, attached macrophage-like cells were harvested with 95 to 99% purity, as evaluated by flowcytometry or immunocytochemistry. These cells showed typical macrophage morphology and were strongly positive for markers of rat macrophages, such as ED-1, ED-3, and OX-41, but negative for cytokeratins and α-smooth muscle actin. They possessed functional properties of typical macrophages, including active phagocytosis of latex beads, proliferative response to recombinant GM-CSF, secretion of inflammatory and anti-inflammatory cytokines upon stimulation with LPS, and formation of multinucleated giant cells. As more than 10^6 cells can be recovered repeatedly from a T75 culture flask at two to three day intervals for more than two weeks, our procedure might implicate a novel alternative to obtain Kupffer cells in sufficient number and purity without complex equipment and skills.

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A Distinct Pro-Resolving Macrophage Subtype Generated By Satiated Uptake Of Apoptotic PMN And Regulated By Resolvins And Glucocorticoids

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During the resolution phase of inflammation apoptotic leukocytes are efferocytosed by macrophages in a nonphlogistic fashion that results in diminished responses to bacterial moieties and production of anti-inflammatory cytokines. Complement receptor 3 (CR3) and pro-resolving lipid mediators promote the engulfment of apoptotic leukocytes by macrophages. Here, we present evidence for the emergence of pro-resolving, $CD11b^{low}$ macrophages *in vivo* during the resolution of murine peritonitis. These macrophages are distinct from the majority of peritoneal macrophages in terms of their functional protein expression profile, as well as pro-resolving properties, such as apoptotic leukocyte engulfment, indifference to TLR ligands, and emigration to lymphoid organs. Notably, we also found macrophages convert from the $CD11b^{high}$ to the $CD11b^{low}$ phenotype upon interaction with apoptotic cells *ex vivo*. In addition, we found that the pro-resolving lipid mediators resolvin (Rv) E1 and RvD1, and the glucocorticoid dexamethasone (Dex) regulated pro-resolving macrophage functions *in vivo*. This regulation culminated in a novel pro-resolving function, namely reducing the apoptotic leukocyte ingestion requirement for $CD11b^{low}$ macrophage generation. These new phenotype and molecular pathway markers define the new satiated-macrophage. Thus, we suggest that satisfying-efferocytosis generates $CD11b^{low}$ macrophages that are essential for complete non-phlogistic containment of inflammatory agents and the termination of acute inflammation.

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Lung Microenvironment Contributes To The Resistance Of Alveolar Macrophages To Toll-Like Receptor Agonists Tolerance

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Injection in animals of endotoxin (lipopolysaccharide, LPS) or other toll-like receptor (TLR) agonists, and *in vitro* activation

of macrophages with these different pathogen-associated molecular patterns (PAMPs) lead to the phenomenon of tolerance and cross-tolerance in response to a second injection or stimulation with these PAMPs. In contrast to any other mononuclear phagocytes, alveolar macrophages from LPS-injected mice fail to develop endotoxin tolerance (*J.Infect.Dis.2004,189,1295*). Alveolar macrophages cultured *in vitro* in the presence of LPS, not only did not develop a state of tolerance to a second activation by LPS, but also were primed to a second activation by other TLR agonists (Pam3CSK4, Poly I:C, CpG). A similar observation was made when the TLR2 ligand was used as a first activator. In order to identify the local parameters that prevent the induction of TLR agonist tolerance in alveolar macrophages, mice were injected with antibodies against GM-CSF, a cytokine known to be present at homeostasis in murine lungs and to prevent the induction of endotoxin tolerance. The use of anti-GM-CSF antibodies partially restored the capacity of alveolar macrophages to develop endotoxin tolerance. Because gamma-interferon (IFNγ) is another cytokine known to prevent endotoxin tolerance, and since we could detect IFNγ in lung homogenates at homeostasis, mice were also pre-treated with anti-IFNγ antibodies and IFNγ receptor KO mice were studied. In both cases, a certain level of tolerance could be observed for alveolar macrophages. While epithelial cells are a well-known source of GM-CSF within the alveolar microenvironment, the source of IFNγ was less known.In order to identify the cellular source of IFNγ, different mutant mice were employed. Tolerization of alveolar macrophages could not be obtained in CD3ε KO and Jα18 KO mice, but was observed in RAG KO, IL-15 KO, RAGγc KO, and □KO mice. These results strongly suggest that NK cells are the source of IFNγ and surprisingly favor the idea that B-lymphocytes also contribute to the specific behavior of alveolar macrophages. Indeed, passive transfer of normal B cells into RAG KO and KO mice prevented the induction of endotoxin tolerance otherwise seen in these animals. The nature of the cross-talk between NK and B-cells is under investigation. Altogether, these results demonstrate that cellular and cytokine microenvironment in the lung prevent alveolar macrophages to be rendered tolerant to TLR agonists. This may explain why lungs are prone to local inflammation in many clinical settings following local, remote or systemic insults.

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Lipoxins and Resolvins Selectively Inhibit LPS-induced TNF Production and Enhance Intracellular Killing of E. coli by Primary Human Macrophages

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Macrophage (mφ) responses to Gram-negative bacteria and bacterial lipopolysaccharide (LPS) are crucial to clearance of infection but may also contribute to clinical complications through dysregulated induction of pro-inflammatory cytokines such as tumor necrosis factor (TNF). LPS-induced inflammatory responses by m π can be modulated by lipoxins (LXs) and resolvins (Rvs), endogenous eicosanoid lipids that enhance resolution of inflammation. We aimed to characterize the ability of LXs and Rvs to modulate inflammatory responses of human m φ to LPS and *Escherichia coliin vitro*. Pre-incubation of THP-1 cell-derived mφ cultured in RPMI supplemented with 10% fetal bovine serum (FBS) with LXs and Rvs for 30 min (0.01 - 1 μ M range) prior to LPS stimulation (10 ng/mL for 4h) showed significant reduction of

LPS-induced TNF production for all compounds at the 0.01 μ M dose (IC_{80} = 10nM). In comparison, the effects of LXs and Rvs on human peripheral blood-derived mφ are more varied, depending on the compound; Pre-incubation of human mφ cultured in RPMI (10% FBS) with LXs and Rvs for 30 min (0.01 - 1 μ M range) prior to stimulation with LPS (10 ng/mL for 4h), showed significant reduction in TNF production with the following potency: aspirin-triggered 17(R)-RvD1 (IC_{50} = 10 nM) > RvD1 (IC_{50} = 100 nM) > LXA_4 and LXB_4 (IC_{50} = 110 nM). In contrast, LPS-induced production of IL-6, an acute-phase response cytokine that speeds resolution of inflammation and induces Th-2 type immune responses, was not reduced. M φ pre-treated with 1 μM 17(R)-RvD1 demonstrated increased killing of internalized live *E. coli* at multiplicities of infection of 10:1 and 50:1 as measured by recovered bacterial colony forming units. LXs and Rvs thus selectively skew human mφ acute cytokine production towards a resolution profile and enhance clearance of live bacteria therefore representing candidate anti-infective/pro-resolving agents to reduce bacteria-driven toxicities.

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Delineate the participation of Zn in LPS induced TLR4 signaling

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Zinc deficiency (ZD) leads to low immunity and ZD patients usually suffer from abnormally frequent infections. The molecular mechanism(s) by which ZD results in lowered immunity is not clear. Recent studies show an unexpected requirement for zinc in TLR4 signaling. TLR4 is a part of innate immune system. It is a receptor that recognizes Lipopolysaccharide (LPS) secreted by bacteria. Recognition initiates intracellular signaling, leading to the expression of proteins that facilitates bacteria and virus elimination. Therefore, delineating the participation of zinc in TLR4 signaling may reveal the molecular pathology of ZD. In this context, we are answering 2 questions: where does zinc participate in TLR4 intracellular signaling, and how does it exert its effects? The processes of TLR4 signaling transduction involve ordered activations of key kinases IRAK1, TAK1, IKK, MKK and then MAPKs. In our study, we monitored the modifications of these components under different zinc availability conditions upon LPS stimulation in macrophage-like RAW 264.7 cells and mice peritoneal macrophages. Our studies revealed that upon LPS stimulation, zinc depletion blocked the phosphorylation of several kinases including IKK, MKK, and MAPKs. But interestingly, upstream kinases IRAK1 was still phosphorylated in the absence of zinc. Moreover, we found IRAK1 was properly ubiquitinated in the absence of zinc, but the subsequent degradation occurred in a zinc dosage dependent manner. Therefore, zinc regulates TLR4 signaling by selectively regulating the modification of specific targets.

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Variation in Protein Tyrosine Kinase and Mitogen-Activated Protein Kinase Signaling Contribute to Differences in Heterophil Responsiveness in a Non-Mammalian System

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Protein tyrosine phosphorylation mediates signal transduction and cellular processes and protein tyrosine kinases (PTKs) regulate virtually all signaling events. The mitogen-activated protein kinase (MAPK) super-families are conserved pathways that convert receptor activation into cellular functions and include extracellular response kinases (ERK), c-Jun N-terminal

kinases (JNK) and p38 MAPKs. Previously conducted *in vitro* and *in vivo* studies using two genetically distinct chicken lines (A and B) consistently show that line A heterophils, the avian equivalent to mammalian neutrophils, are functionally more responsive and produce a differential cytokine/chemokine profile when compared to line B heterophils. Additionally, this increased *in vitro* responsiveness translates to increased resistance to bacterial and protozoan challenges. Therefore, we hypothesize that the differential responses between the two lines of chickens result from distinctive MAPK signaling that mediate the heterophil responses. Heterophils from lines A and B were isolated from day-old chickens and total PTK, p38, JNK, and ERK levels quantitated following interaction with *Salmonella enteritidis* (SE). Control and SE-treated heterophils from line A chickens had more ($p \leq 0.05$) total PTK activation compared to line B heterophils and increased ($p \leq 0.05$) levels of p38 protein. However, line B heterophils had higher ($p \leq 0.05$) JNK levels. There were no differences in ERK between control and activated heterophils for either line. AP-1 and NF-κB transcription factor families were also examined and c-jun and p50, respectively, were the only members that were different between the two lines and both were up-regulated in line A compared to B. These data indicate increased responsiveness of line A chickens and/or their heterophils is mediated, in large part, by an increased ability to initiate phosphorylation of PTKs leading to the specific activation of the p38 MAPK pathway, and activation of specific transcription factors, all of which directly affect the innate immune response.

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TLR2 Expression is Increased During In Vivo Transmigration of Human Neutrophils and Results in a Primed TLR Response

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Neutrophils are important players in innate immunity and arrive at inflammatory sites after transmigration into the tissue from circulation. Tissue neutrophils have an altered surface flora of receptors, in part due to degranulation of intracellular receptor-storing granules, and are primed (hyper-responsive) to subsequent stimulation. Priming has been mostly investigated in relation to chemoattractants, the receptors for which are upregulated to the cell surface by degranulation. Less is known about the pathogen-associated molecular patterns (PAMP)-binding Toll-like receptors (TLRs) on *in vivo* transmigrated neutrophils, both regarding expression levels and whether transmigrated cells are functionally primed for PAMP activation in the tissues.

In vivo transmigrated human neutrophils were obtained using a skin chamber technique and compared to peripheral blood neutrophils from the same donor. The transmigrated neutrophils displayed elevated levels of various receptors (e.g., complement receptors, chemoattractant receptors and cytokine receptors) including TLR2, as compared to peripheral blood neutrophils. Potential mechanisms behind the upregulation of TLR2 during *in vivo* transmigration could be *de novo* TLR2 synthesis and/or a result of mobilization of TLR2 containing granules. Measuring IL-8 production, transmigrated neutrophils were hyper-responsive to various TLR2-binding PAMPs, including Pam3CSK4.This indicates that upregulation of TLR2 during transmigration renders neutrophils primed to PAMP activation in the tissues.

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Binge Alcohol Drinking Modulated TNFa Promoter Leading to Low TNFa Production in Macrophages via Lipid Raft-dependent Mechanism

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The consequences of binge drinking alcohol abuse on the liver and inflammation are not fully understood, however a role for impaired macrophage functions was suggested. Here we aimed to identify the mechanism of the inhibitory effects of alcohol binge on TNFα production in liver macrophages, Kupffer cells (KCs).

Methods. C57Bl6 mice received ethanol (5g/kg ig) or isocaloric/isovolumetric maltose-dextrin pair-feed for 3 consecutive days, LPS (1mg/kg ip) or live E.coli with/without KC depletion with clodronate-containing liposomes. Serum ALT was assayed biochemically, TNF α gene by PCR and protein by ELISA, DNA binding by EMSA and ChIP. Kupffer cells (KC) we isolated by enzymatic digestion and gradient centrifugation; RAW264.7 cells were sub-cultured every 3 days.

Results. Alcohol binge, but not pair-feeding, and LPS challenge lead to elevation of serum ALT; alcohol binge augmented LPS-induced serum ALT increase. Exposure to live E.coli lead to significantly higher ALT, decreased serum TNF α and increased mortality in alcohol- or clodronate-treated animals compared to controls, indicating that alcohol impairs immune defense in a macrophage-dependent manner. Alcohol binge impaired LPS-induced production of TNF α in the serum (ELISA), in whole liver (PCR) and in isolated KCs (PCR and ELISA). Alcohol-exposed KCs exhibited increased membrane fluidity, suggestive of lipid raft impairment; membrane stabilization with cholesterol treatment abolished the alcohol-induced increase in membrane fluidity and partially restored the LPS-induced TNF α production. Alcohol-exposed RAW cells, as models of KCs, had lower activation of MAPK, compared to alcohol-naïve cells. LPS-induced activation of NFκB transcription factor was attenuated in alcohol-exposed RAW cells, while AP-1 activation was preserved compared to controls. Lipid raft solubilization with MβCD or membrane stabilization with cholesterol treatment did not affect LPS-induced activation of AP-1, suggesting that AP-1 activation is lipid raft-independent. Inhibition of AP-1 (nordihydroguaiaretic acid) partially rescued alcohol-induced impairment of NFκB binding to TNF α promoter and increased TNF α protein production. Finally, alcohol-exposed RAW cells showed decreased NFκB and increased AP-1 binding to the TNFa promoter compared to controls; the NFkB was composed of p50/p65 and AP-1 of c-Jun/c-Fos. Collectively, these data suggested that alcohol binge inhibits TNF α production in Kupffer cells via lipid-raft-dependent impairment of NFκB while facilitating raft-independent AP-1 binding to TNF α promoter.

In conclusion, we report that alcohol inhibits the TNF α production in macrophages by modulating the ratio of NFκB / AP-1 transcription factors at the TNF α promoter in a lipid raft-dependent manner. Our novel data may aid new therapeutic strategies of alcohol-binge induced liver inflammation.

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Development of a Novel Assay that Reveals Striking Inter-Individual Differences in Ability of Endotoxin to form a Bioactive Complex with MD-2 in Plasma of Hematopoietic Stem Cell Transplant Patients

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Hematopoietic stem cell transplantation (HSCT) is a therapy used to cure benign and malignant conditions of the bone marrow but may cause serious treatment-related toxicities including graft-versus-host disease (GVHD). Virtually all patients become endotoxemic and ca. 30-60% later develop signs of acute GVHD, raising the possibility that individuals may differ in their

response to endotoxemia. We speculated that the pro-inflammatory effect of endotoxemia and an increased risk of acute GVHD could depend upon inter-individual differences in the ability of blood-borne endotoxin to be delivered to MD-2, the principal endotoxin-binding component of the pro-inflammatory Toll-Like Receptor (TLR) 4. To test this hypothesis, we have developed a novel, highly sensitive, and quantitative assay that measures the extent to which uniformly labeled (25,000cpm/pmol), purified ^{3}H meningococcal lipooligosaccharide (LOS) forms a complex with added recombinant *his*$_6$-MD-2 in patient vs. healthy, control plasma. Formation of ^{3}H LOS-*his*$_6$-MD2 complex was measured by capture using Ni^{++} chelating resin. There was virtually no capture of ^{3}H LOS without addition of *his*$_6$-MD-2, but up to 40% capture using as little as 250 pg ^{3}H LOS/ml when *his*$_6$-MD-2 was added. There were striking differences in ^{3}H LOS capture in plasma from different patients at baseline (i.e., before beginning HSCT treatments) (range: 0% to 275%; mean: 59 ± 61% (SD); (n = 43) of ^{3}H LOS capture in a healthy, control plasma used as reference). Plasma samples from healthy donors showed much less inter-individual variability (range: 53% to 173%; mean: 109 ± 38.6% (SD); (n = 11). The sensitivity and speed of this new assay may help identify HSCT patients most at risk for the pro-inflammatory effects of endotoxemia.

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Oncostatin M has the anti-inflammatory effect of decreasing IL-1β secretion by human synovial fibroblasts

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Objectives. Accumulating evidence indicates that the cytokine oncostatin M (OSM) is a mediator in the attenuation of inflammation and is provided endogenously at the inflammatory site, notably by polymorphonuclear neutrophils (PMNs). We assessed the impact that OSM may have on the activation of human synovial fibroblasts (HSFs), key contributors to the progression of the inflammatory response in rheumatoid arthritis.

Methods. Cytokine mRNA and protein levels, quantified respectively using real-time PCR and ELISA, were examined in three different contexts: 1. HSFs were incubated for 24h with supernatants of LPS/GM-CSF-stimulated PMNs with or without anti-OSM neutralizing antibody; 2. HSFs were stimulated directly with LPS/GM-CSF in the presence or not of recombinant human OSM; 3. *In vivo* impact of recombinant OSM was investigated in LPS-stimulated murine dorsal air pouches, a model of synovial environment.

Results. OSM neutralization in the supernatants of stimulated PMNs yielded results in line with previous studies performed with the recombinant protein. We thus observed that OSM decreases mRNA expression of HSF-derived inflammatory factorsCXCL-8 and GM-CSF, while increasing that of IL-6 and CCL-2. We note with interest that the neutralization and recombinant approaches both indicated that OSM is a potent inhibitor of the expression of mRNA and protein in the case of IL-1β, a key initiator of inflammatory processes. In the murine dorsal air pouch, mouse recombinant OSM reduced TNF α and IL-1 β mRNA in both the migrated leukocytes and resident lining cells, as well as cytokine levels in the exudates.

Conclusions. This study further emphasizes the immunomodulatory functions of OSM, by documenting for the first time the inhibition of HSF-generated IL-1 β PMNs being a significant source of OSM and a major cell type present in the inflamed synovial fluid of rheumatoid arthritis patients, PMN-derived OSM could help

to limit the magnitude and duration of inflammatory responses.

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Inhibitory effect of 10-hydroxy-trans-2-decenoic acid on IFN-γ-induced nitric oxide production via inhibiting TNF-α autocrine stimulation

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10-Hydroxy-trans-2-decenoic acid (10H2DA) is a major lipid component of royal jelly, which is a special food for honey bee queen. Since 10H2DA is specifically found in RJ, it is expected to have some biological activities. Several pharmacological activities, including antibiotic, anti-tumor, estrogenic, neurogenesis and angiogenesis activity, have been reported for 10H2DA. We previously investigated the effect of 10H2DA on the expression of various genes in LPS-stimulated macrophage cells and found that 10H2DA inhibits LPS-induced IL-6 production. In this study, we examined the effect of 10H2DA on IFN-γ-induced nitric oxide (NO) production in RAW264 cells. IFN-γ-induced NO production was inhibited by 10H2DA, dose-dependently. Luciferase reporter gene assay showed that 10H2DA significantly inhibited the LPS-induced promoter activity of inducible NO synthase (iNOS), suggesting the transcriptional inhibition of iNOS gene. The phosphorylation of signal transducer and activator of transcription-1 (STAT-1), which is a transcription factor activated in IFN-γ stimulation, was not affected by 10H2DA. Farther, 10H2DA affected neither of the nuclear translocation of STAT1 nor IFN-gamma activation site (GAS) promoter activation by IFN-γ stimulation. Moreover, IRF-1 induction by IFN-γ was also not affected by 10H2DA. On the other hand, IFN-γ-induced TNF-α production and nuclear factor (NF)-κB activation were significantly inhibited by 10H2DA. Furthermore, TNF-α-induced NF-κB activation was also inhibited by 10H2DA, dose-dependently. It has been reported that IFN-γ-induced TNF-α production followed by activation of NF-κB is essential for NO production in stimulation with IFN-γ. Together, 10H2DA inhibited IFN-γ-induced NO production via inhibiting TNF-α production and NF-κB activation without affecting STAT-1 activation or IRF-1 induction. 10H2DA might be a candidate for a specific inhibitor for NF-κB activation in IFN-γ stimulation.

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Albumin is a donor of endotoxin monomers to MD-2, facilitating CD14-independent activation of Toll-Like Receptor 4

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Infection by Gram-negative bacteria (GNB) typically elicits a rapid and robust innate immune response. This is partially mediated by the recognition of GNB-derived endotoxin (E), which is a unique and abundant glycolipid located on the outer leaflet of the GNB outer membrane, by host immune cells. Potent host response to E depends on the ordered interaction of E with lipopolysaccharide binding protein (LBP), CD14 , MD-2 and Toll-like receptor 4 (TLR4). CD14 is required for maximal host sensitivity to E by facilitating the efficient transfer of E monomers to MD-2 and MD-2 ·TLR4. However, activation of MD-2(·TLR4) by E is possible in the absence of CD14 through unknown and apparently less efficient mechanism(s). We now show that incubation of purified E aggregates ($M_r \geq 20$ million) in PBS with $\geq$ 0.1% albumin in the absence of the divalent cations Ca^{2+} and $Mg^{2+,}$ yields monomeric E·albumin complexes (M_r ~70,000)

that can transfer E monomer to MD-2 and MD-2 ·TLR4. E·albumin induces MD-2·TLR4-dependent, CD14-independent cell activation, although at ~10 fold lower levels than cell activation induced by monomeric E·sCD14 complex. This difference in potency parallels differences in the rate of E monomer transfer to MD-2 from E·albumin vs. E·CD14, and differences in the apparent K_d of E monomer transfer to MD-2 (~4.0 nM vs. ~0.8 nM, respectively, from E·albumin vs. E·CD14). Our findings suggest for the first time a possible mechanistic basis for CD14-independent cell activation by E. E·CD14 and E·albumin complexes should provide valuable reagents for future studies of MyD88-dependent (CD14-independent) and TRIF-dependent (CD14-dependent) TLR4 signaling by E.

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Neuraminidase pre-treatment sensitizes mice to LPS-induced acute lung injury

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Background: Seasonal and pandemic influenza may be complicated by acute lung injury. Since we previously reported that removal of sialic acid residues primed leukocytes to respond to LPS stimulation *in vitro*, we speculated that NA treatment can sensitize the host to an enhanced inflammatory response upon exposure to a TLR ligand, like LPS. We therefore developed a murine acute lung injury model to study the effect of desialylation on LPS-induced inflammatory responses.

Methods: Outbred mice were administered intratracheally (i.t.) *clostridial* neuraminidase (NA, 100 mU) or PBS and challenged i.t. 30 minutes later with LPS (5 mg) or PBS. The leukocyte numbers and cytokine concentrations in the bronchoalveolar lavage fluid (BALF) at different time points and wet and dry weights of lung tissues were determined. The total RNA was extracted from lung tissues for quantitative real-time PCR analysis.

Results: LPS induced PMN transmigration into alveoli (2.29 ± 0.63 x10^5 vs. PBS 0.32 ± 0.4 x10^5), and higher wet to dry ratio of lung tissue (1.39 ± 0.03 vs. PBS 1.23 ± 0.02). Within 30 min , NA desialylated bronchiole and alveolar surfaces by lectin histochemistry. Compared to LPS challenge alone, NA pre-treatment with LPS challenge increased PMN number (4.53 ± 0.83 x10^5) in BALF and wet to dry ratio (1.61 ± 0.03). The PMN recruitment and wet to dry ratios with NA alone did not differ from those of PBS. Elevation of mRNA for chemokines, such as CCL2, CXCL1, CXCL2, CXCL3, and TNF in lung homogenates occurred within 30 minutes after NA treatment. Consistently, NA alone induced KC, LIX, and TNF-α production in BALF, as did LPS. But NA pre-treatment increased IL-1β (4.1 fold) and TNF-α (1.7 fold) production after LPS challenge.

Conclusion: NA pretreatment sensitizes the host to LPS induced recruitment of PMNs, proinflammatory cytokines, and severe lung injury. Thus, desialylation that may occur during influenza infection may lead to more severe responses to TLR ligands found with bacterial superinfection.

Significance: The "reprogramming" of immune responses by desialylation may contribute to severe lung injury with secondary bacterial infections.

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An antimicrobial cathelicidin peptide LL-37 suppresses the LPS-induced endothelial cell apoptosis

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[Aim] In severe sepsis or septic shock, endothelial cell apoptosis is induced in lung or liver, and eventually triggers organ dysfunction. Previously, we reported that an antimicrobial human cathelicidin peptide LL-37 protects mice from lethal endotoxin shock by inhibiting inflammatory cytokine production by mononuclear phagocytes. Here, to further evaluate the role of LL-37 in endotoxin shock, we investigated the effect of LL-37 on the LPS-induced endothelial cell apoptosis in vitro and in vivo.
[Methods] Apoptosis of endothelial cells was induced by incubating lung-derived human microvascular blood vessel endothelial cells (HMVEC-L) with LPS and cycloheximide (CHX). Apoptosis was determined by Annexin V/propidium iodide and TUNEL stainings, and the LPS-binding to HMVEC-L was analyzed with fluorescence-labeled LPS by flow cytometry. Concomitantly, the effects of LL-37 on the apoptosis and LPS-binding were examined. To determine the involvement of CD14 and TLR4 in the LPS-induced apoptosis, HMVEC-L were incubated with LPS/CHX in the presence of anti-CD14 or anti-TLR4 mAb. In addition, LL-37 was administered to D-galactosamine-sensitized endotoxin shock mice, and its effect on the endothelial cell apoptosis was evaluated in liver by double staining with anti-CD31 mAb and TUNEL.
[Results & Conclusions] LL-37 dose-dependently suppressed the LPS/CHX-induced apoptosis of HMVEC-L, and inhibited the LPS-binding to the cells. In addition, anti-CD14 and anti-TLR4 mAbs significantly inhibited both the LPS-induced apoptosis and LPS-binding. These observations suggest that LL-37 protects HMVEC-L from the LPS-induced apoptosis possibly via the inhibition of CD14/TLR4-mediated LPS signaling. Furthermore, LL-37 suppressed the apoptosis of hepatic endothelial cells in D-galactosamine-sensitized endotoxin shock mice. Thus, LL-37-induced suppression of endothelial cell apoptosis may be involved in the protection of mice from lethal endotoxin shock.

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Stimulation of Toll-like receptors leads to proteasome-dependent downregulation of TRAF6 through interaction with IRAK-1

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TRAF6 is an E3 ubiquitin ligase and plays a crucial role in signal transduction of the Toll-like receptor (TLR)/interleukin-1 receptor superfamily. It has been reported that TRAF6 catalyzes the formation of unique Lys^{63}-linked polyubiquitin chains, which does not lead to proteasome-mediated degradation. In this study, we found that stimulation of J774.1 cells with various TLR ligands led to decrease in TRAF6 protein with delayed kinetics compared to IκBα degradation. This decrease in TRAF6 was inhibited by proteasome inhibitors MG-132, lactacystin and N-acetyl-leucyl-leucyl-norleucinal. Among MyD88, IRAK-4, IRAK-1, TRAF6 and IKKβ intracellular signaling molecules for TLR, expression of only IRAK-1 led to TRAF6 downregulation in HEK293 cells. The amount of TRAF6 expressed either transiently or stably was also reduced by co-expression of IRAK-1 with no cleavage products of TRAF6 detected. The levels of either an N-terminal deletion mutant or a ubiquitin ligase-defective mutant of TRAF6 were not affected by IRAK-1 expression. Downregulation of TRAF6 required the TRAF6-binding site (Glu^{544}, Glu^{587}, Glu^{706}) of IRAK-1 but not its catalytic site (Asp^{340}). Upon IRAK-1 transfection, no significant TRAF6 ubiquitination was detected. Instead, TRAF6-associated IRAK-1 was ubiquitinated with both Lys^{48}- and Lys^{63}-linked polyubiquitin chains. TRAF6 downregulation was significantly inhibited by co-expression of the E3 ubiquitin ligase Pellino 3, whose Lys^{63}-linked polyubiquitination on IRAK-1 is reported to compete with Lys^{48}-linked IRAK-1 polyubiquitination. These results indicate that stimulation of TLRs leads to proteasome-dependent downregulation of TRAF6 and suggest that TRAF6 associated with the ubiquitinated IRAK-1 is degraded together with IRAK-1 by the proteasome.

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Mechanistic role of miR-146a in TLR ligand-induced differential cross-regulation of TLR signaling

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Human toll-like receptors (TLRs) are critical sensors for microbial components leading to the production of proinflammatory cytokines. Monocytes pretreated with LPS exhibit a state of hyporesponsiveness to both LPS and other TLR ligands, referred to as cross-tolerance, which play a broader role in innate immunity. To date, LPS-induced cross-tolerance has not been examined regarding microRNA expression kinetics. **Objective**: To examine the role of miR-146a in TLR ligand-induced cross-tolerance. **Methods**: A cross-tolerance cell model using monocytic cell line THP-1 was adapted. Cells were incubated/primed with low dose of a TLR ligand for 18 h and then challenged with various doses of different TLR ligands for an additional 5 h. Culture supernatants were assayed for TNF-α production. **Results**: THP-1 treated with various TLR ligands showed a continuous amplification of miR-146a over 24 h, which is inversely correlated to TNF-α production. In contrast, inhibition of miR-146a showed a reciprocal effect. Strikingly, in LPS-tolerized THP-1, only miR-146a showed a continuous overexpression, suggesting its crucial role in cross-tolerance. Similarly, peptidoglycan-primed THP-1 cells showed homologous tolerance associated with miR-146a up-regulation. Subsequently, interchangeable differential cross-regulation was observed among non-LPS ligands. TLR2 and TLR5 ligands showed both homologous and heterologous tolerance correlated with miR-146a overexpression. More importantly, inflammatory responses to TLR4, TLR2, and TLR5 ligands were reduced in cells knockdown in miR-146a targets IRAK-1 or TRAF6, suggesting the regulatory effect of miR-146a on these TLRs signaling. Transfection of miR-146a into THP-1 cells caused reduction of TNF-α production, mimicking LPS-induced cross-tolerance. Aside from individual ligands, a whole bacterial challenge in LPS-primed THP-1 monocytes was accompanied by less TNF-α production, which is conversely correlated to miR-146a expression. **Conclusion**: Our studies have demonstrated that miR-146a plays a crucial role for *in vitro* monocytic cell-based TLR-induced cross-tolerance.

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Stimulus-specific Post-transcriptional Regulation of IκB-ζ in Innate and Adaptive Immune Responses.

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IκB-ζ is a nuclear IκB family protein that is barely detectable in unstimulated cells and is robustly induced by various ligands for Toll-like receptors (TLRs). Our previous studies indicated that IκB-ζ is a crucial transcriptional regulator upon stimulation of TLRs: it plays an essential role for induction of the secondary response genes such as interleukin (IL)-6 via association with nuclear factor (NF)-κB . The induction of IκB-ζ is also elicited by IL-1β , but not by tumor necrosis factor (TNF)-α . We have shown that the induction of IκB-ζ requires stimulus-specific mRNA stabilization via a 165-nt element in the 3'-untranlated region of IκB-ζ mRNA in addition to transcriptional activation by NF-κ B . Here, we found that IκB-ζ induction was also elicited via the 165-nt element-dependent post-transcriptional regulation on the B cell antigen receptor (BCR) stimulation. The activity of a luciferase reporter that constitutively expresses a fusion mRNA harboring the element was upregulated upon crosslinking BCR. Interestingly,

the upregulation was suppressed on co-crosslinking BCR with the inhibitory Fc-receptor IIB. The 165-nt element-mediated regulation was activated by overexpression of MyD88, a common adaptor for TLR/IL-1 receptor, but not that of the TNF receptor adaptor TRADD although both adaptors activated the pathway culminating in NF-κB and MAP kinase activations. Furthermore, it was shown, by using NF-κB inhibitors, that the post-transcriptional regulation of IκB-ζ is independent of NF-κB activation in both macrophages and B cells. miRNA did not appear to be involved in the regulation: the IκB-ζ induction pattern was not affected in macrophages lacking the miRNA processing enzyme Dicer. These results indicated that the post-transcriptional regulation of IκB-ζ was specifically activated both in the innate and adaptive immune responses via a distinct pathway from the one leading to NF-κB activation.

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Gene-specific translational regulation by 10-hydroxydecanoic acid in LPS-stimulated murine macrophage RAW264 cells

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We investigated the effect of 10-hydroxydecanoic acid (10HDA), which is one of the major fatty acid components of royal jelly, in LPS-stimulated RAW264 murine macrophage cell line. 10HDA did not affect LPS-induced production of proinflammatory cytokines (TNF-α, IL-6) and chemokines (MCP-1, MIP-1/2, IP-10). However, 10HDA significantly inhibited nitric oxide (NO) production. In reporter gene assay, inducible NO synthase (iNOS) promoter activity was inhibited by 10HDA. Moreover, ISRE and NF-κB binding site, which are essential cis-elements in iNOS promoter, were activated by LPS, and 10HDA inhibited the activation of ISRE but not NF-κB. In RT-PCR analysis, LPS-induced IFN-β production, which is responsible for ISRE activation in LPS stimulation, was not affected by 10HDA. Then, we examined the effect of 10HDA on IFN-β signaling. 10HDA inhibited IFN-β-induced NO production. However, LPS-induced phosphorylation of signal transducer and activator of transcription (STAT)-1 and 2, and mRNA transcription of interferon regulatory factor-1 (IRF1) were not affected by 10HDA. Interestingly, we found that the LPS-induced expression of IRF-1 protein was significantly down regulated by 10HDA, suggesting that 10HDA inhibited IRF1 mRNA translation. To further investigate the effect of 10HDA on mRNA translation, polysomal mRNA was isolated and the amount of mRNA in the polysomal fraction was determined. mRNA level of IRF1, but not TNF-α and MIP-1, in the polysomal fraction isolated from 10HDA-treated cells was significantly reduced in comparison to that from untreated cells. Furthermore, phosphorylation of Akt, which is suggested to be responsible for IFN-induced mRNA translation of IFN-target genes, was significantly inhibited by 10HDA. Together, these results suggest that 10HDA specifically inhibits IRF1 mRNA translation, then reduces iNOS gene induction and decreases NO production. This inhibitory effect may be mediated by inhibition of Akt phosphorylation.

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Macrophage Phenotype in Sterile Wounds in TLR4-Incompetent Mice

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Previous work in TLR4-incompetent mice (C3H/HeJ and TLR4 KO) demonstrated that extracellular fluids of early sterile wounds from these animals contained less CCL3 and CCL5 and more CCL17 than those from control mice (C3H/HeOuJ or C57BL6). The number and differential counts of inflammatory cells in wounds of animals of all strains were the same. The potential impact of differences in wound chemokine concentrations on the phenotype of macrophages from wounds in C3H/HeOuJ and C3H/HeJ mice was examined and is reported here. Methods: Wound cells were recovered one

day after subcutaneous implantation of polyvinyl alcohol sponges in /HeOuJ and /HeJ mice. Isolated cells were analyzed by flow cytometry. Results: The frequency of Gr-1^{high} wound macrophages was lower in /HeJ vs. controls (48% vs. 73%). This was not due to a decreased number of circulating Gr-1^{high} monocytes in the /HeJ mice. Wound fluid content of CCL2 and CX3CL1, chemokines thought to respectively recruit Gr-1^{high} and Gr-1^{low} monocytes, was the same in both mouse strains. Conclusions: Results confirm and extend evidence supporting a role for TLR4 in the recruitment of different macrophage precursors to sterile wounds.
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Immunoregulatory functions of an anti-infective peptide and its dependency on the PI3-Kinase pathway

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The increased prevalence of antibiotic resistance has promoted the search for new therapeutics against bacterial infections. Innate defence regulators (IDRs), synthetic derivatives of natural host defence peptides, are being developed as anti-infective agents due to their ability to modulate innate immune responses. IDR-1002, a derivative of bovine bactenecin, was shown to be protective in murine models of *Staphylococcus aureus* and *Escherichia coli*. Although this protection correlated with an increased chemokine production and recruitment of leukocytes to the site of infection, the mechanism by which IDR-1002 regulates the immune response, and affords protection against bacterial infection, is still unknown. We found that IDR-1002 utilizes the PI3-Kinase, a complex signalling pathway that regulates inflammation and immunity, for many of its immunomodulatory effects. IDR-1002 suppresses inflammatory cytokine production in human blood mononuclear cells (hBMC) stimulated with lipopolysaccharide (LPS), correlating with a suppression of MAP-Kinase signalling in human monocytes. The suppressive effect on LPS-induced cytokine production and MAP-Kinase signalling by IDR-1002 is partially dependent on the PI3-Kinase pathway. In addition, IDR-1002 induces surface expression of β-2 integrins on human monocytes in a PI3-Kinase dependent manner. The ability of IDR-1002 to promote β-2 integrin expression on monocytes correlates with its ability to promote monocyte adhesion to extracellular matrix components, an essential step in the recruitment and migration of monocytes to the site of infection. Understanding how IDRs utilize the PI3-Kinase pathway to elicit their broad-ranged regulatory functions is essential to the development of novel anti-infective therapeutics.
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Interaction of Adaptor Proteins with the TIR domain of Toll-Like Receptor-4

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Toll-like receptors (TLRs) are key regulators of the immune system, recognizing both microbial molecules ranging from bacterial cell surface components to viral genomes, and a wide variety

of host molecules involved in signaling danger. TLR-4 is the best characterized member of the TLR family which, like all other members, initiates intracellular signaling with the recruitment of TIR-domain-containing adaptor proteins to the cytoplasmic domain. Four TIR (Toll/Interleukin-1 Receptor) domain-containing adaptors are involved in propagating TLR signaling: MyD88, Mal (also called TIRAP), TRAM and TRIF. These adaptors link activated TLRs with 'downstream' kinases of the IL-1 receptor-associated kinase and mitogen-activated protein kinase families. Activation of these enzymes leads to the activation of two distinct signaling pathways involving transcriptional regulators such as NFκB, AP-1 and several interferon regulatory (IRFs), which induce hundreds of genes involved in immune defense. TLR-4 is activated by exposure to lipopolysaccharide (LPS) derived from the outer membrane of Gram negative bacteria, and subsequently induces downstream signaling by lingand-induced dimerization. Recent studies show that this homodimerization can cause conformational changes in the receptor leading self-association of the cytoplasmic TIR signaling domain, providing a new scaffold that is able to bind downstream signaling adaptor proteins. Structure modeling studies of the TLR-4 TIR domain suggest that both Mal and TRAM adaptors bind at a two symmetry-related sites at the homodimer surface (Miguel et al 2007). This study aims to investigate the nature of adaptor recruitment to the TLR-4 TIR domain, using FRET (Fosters Resonance Energy Transfer), luciferase reporter assays and protein-protein binding assays.

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LILRA2 modulates LPS-mediated cytokine production and phagocytosis in Peripheral Blood Monocytes

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Objective: The activating immunoglobulin-like receptor A2 (LILRA2) is expressed on the surface of cells that play key role in the innate immune system such as monocytes, macrophages and neutrophils. Over-expression of LILRA2 on macrophages is strongly associated with lepromatous leprosy, a more detrimental form of the disease characterized by excessive Th2 immune response. However, the functions of LILRA2 and how it modulates monocyte activation remain unknown. Our aim was to examine how LILRA2 regulates monocyte's function, particularly in the presence of LPS.

Methods: Primary monocytes were optimally activated by anti-LILRA2 antibody cross-linking and/or simultaneously stimulated with LPS (10ng/ml), and the levels of cytokines production were analyzed using a multiplex assay. To assess phagocytosis, fluorochrome conjugated IgG-coated micro-beads or enhanced green fluorescent protein (EGFP)-expressing *Escherichia coli* were added into monocytes, the percentage of phagocytosis was analyzed by flow cytometry. TLR4 and CD14 expression were analyzed by Real-time PCR and flow cytometry.

Results: We show optimal activation of monocytes by LILRA2 cross-linking failed to induce IL-12 and MCP-1 production that were strongly up-regulated by LPS. Interestingly, LILRA2 cross-linking on monocytes induced similar levels of IL-6, IL-8, G-CSF and MIP-1α but lower levels of TNFα, IL-1β, IL-10 and IFNγ to those stimulated with LPS. In addition, cross-linking of LILRA2 on monocytes significantly decreased phagocytosis of IgG-coated micro-beads, while had no effect on phagocytosis of *Escherichia coli*. unexpectedly, simultaneous co-stimulation of monocytes through LILRA2 and LPS, or pre-activation of monocytes via LILRA2 followed by LPS treatment led to significantly lower levels of TNFα, IL-1β, IL-12 production as compared to LPS alone. However, there was no effect on level of IL-6, IL-10 and IFNγ production. Furthermore, LILRA2 cross-linking on monocyte caused significant down-regulation of TLR4 and CD14 mRNA and protein, indicating LILRA2-mediated suppression of LPS responses

might be in part through regulation of these receptors.

Conclusions: We provide evidence that LILRA2-mediated effects on monocytes significantly differ from that of LPS, and LILRA2 strongly and selectively regulates LPS-mediated monocyte activation and IgG-dependent phagocytosis. Down-regulation of IL-12 production after LILRA2 activation may prompt to a Th2 immune response observed in lepromatous leprosy.

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Crosstalk between the toll-like receptor-4 (TLR-4) and angiotensin-II in the activated hepatic stellate cells

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[Aim] The role of Toll-like receptors (TLRs) and TLR ligands in the pathophysiology of liver fibrosis has been proposed. Angiotensin-II (AT-II) induces the proliferation and transforming growth factor-b (TGF-b) production in the hepatic stellate cells (HSC), which plays a pivotal role in the liver fibrogenesis. It has been reported that AT-II up-regulated TLR-4 and LPS-induced several gene expressions in several types of cells. However, the possible interaction between AT-II and TLR-4 in the liver fibrosis development has not been elucidated yet. The aim of the current study was to examine the cross talk between AT-II and TLR-4 in the activated HSC, and possible mechanisms involved in it.

[Methods] We used the rat activated HSC line (T-6) to elucidate the effect of AT-II on several indices including TLR-4, MyD88, NF-kb, and TGF-b expressions. The effect of AT-II and LPS on the proliferation of HSC was also examined.

[Results] AT-II significantly augmented the TLR-4 mRNA expression in a dose- and time-dependent manner, and these effects were almost attenuated by ARB. We also observed that AT-II markedly increased the mRNA of MyD88 and NF-kb, and these effects were also suppressed by ARB. The LPS-induced augmentaion of MyD88 and NF-kb expression were synergistically increased with the treatment of AT-II. The AT-II-induced augmentaion of TGF-b expression was synergistically increased with the treatment of LPS. The proliferation of activated HSC was significantly stimulated by AT-II in a dose-dependent manner but not by LPS. ARB attenuated these effects of AT-II on MyD88, NF-kb and proliferation of HSC

[Conclusion] AT-II exerts a close cross talk with innate immunity system in the activated hepatic stellate cells. AT-II and TLR-4 signaling cascades may co-ordinate in the progression of liver fibrosis.

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Enhancement of Toll-Like Receptor 4 activation by a TLR4 antagonist

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Monomeric complexes of hexaacylated meningococcal lipooligosaccharide (LOS) bound to wild-type (wt) human MD-2 (LOS·MD-2^{wt}) are potent TLR4 agonists. In contrast, the mutant complex LOS·MD-2^{F126A}, is a potent TLR4 antagonist with <1% the agonist activity of LOS·MD-2^{wt}. LOS·MD-2^{wt} and LOS·MD-2^{F126A} as well as MD-2^{wt} and MD-2^{F126A} without bound LOS bind to *Flag*-TLR4 ectodomain (TLR4-ecd) with virtually identical affinity as judged by: 1) Scatchard analysis of binding of [^{3}H]LOS·MD-2^{wt} and of [^{3}H]LOS·MD-2^{F126A} to TLR4-ecd (k_d of 98 and

89 pM, respectively); and 2) similar dose-dependent inhibition of [^{3}H]LOS·MD-2^{wt} binding to TLR4-ecd by added unlabeled LOS·MD-2^{wt}, LOS·MD-2^{F126A}, MD-2^{wt}, and MD-2^{F126A}. Added MD-2^{wt} or MD-2^{F126A} produced closely similar dose-dependent inhibition of activation of HEK/TLR4 cells by low concentrations (20 pM) of LOS·MD-2^{wt}, with nearly complete inhibition at 1 nM MD-2 (TLR4-reactive monomer). At 1 nM, LOS·MD-2^{F126A} also inhibited TLR4 activation by 20 pM LOS·MD-2^{wt}. Remarkably, however, at lower concentrations (60-180 pM) that were sub-saturating for TLR4, added LOS·MD-2^{F126A}, but not sMD-2 alone, increased TLR4 activation induced by 20 pM LOS·MD-2^{wt}. Increased TLR4 activation by 20 pM LOS·MD-2^{wt} + 60-180 pM LOS·MD-2^{F126A} (vs. 20 pM LOS·MD-2^{wt} alone) was statistically significant ($p < 0.001$) and as great as that induced by doubling the amount of added LOS·MD-2^{wt} to 40 pM. These findings suggest that occupation of TLR4 by LOS·MD-2^{F126A}, while insufficient alone to induce increased TLR4 dimerization and activation, can induce formation of hetero-dimeric ternary complexes (i.e., LOS·MD-2^{wt}·TLR4/ LOS·MD-2^{F126A}·TLR4) that are active and result in increased TLR4 activation when levels of TLR4-activating wt LOS·MD-2 complexes are limiting. These findings seem most consistent with a model in which the magnitude of TLR4 activation is determined by the surface density of potentially interacting ternary (ligand·MD-2·TLR4) complexes and the probability of ternary complex interaction can be modulated by the structural properties of the ligand (endotoxin)·MD-2 complex.

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TLR4 and sphingosine 1-phosphate receptors cooperate to increase adhesion molecule expression and leukocyte-endothelial adhesion

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Background and aims: Toll-like receptors (TLR) are innate receptors that can sense molecular patterns present in pathogens as well as endogenous molecules. We have previously demonstrated in human monocytes - macrophages the attenuation of TLR2 signalling by the inflammatory lipid mediator sphingosine-1-phosphate (S1P) through receptors 1 and 2, which could explain some of the S1P anti-atherogenic effects. Since leukocyte adhesion to the endothelium via adhesion molecules such as ICAM-1, VCAM-1 and E-selectin is an important event in the initiation of atherosclerosis , our goal was to investigate whether the TLR-S1P receptors crosstalk had an impact on leukocyte-endothelial adhesion .
Methods: Human endothelial cells from arterial (HAEC) and venous (HUVEC) origins were treated with a combination of S1P and TLR ligands. The expression of adhesion molecules was analyzed by Western blot and flow cytometry. The adhesion of human peripheral blood mononuclear cells to endothelial cell monolayer was evaluated using a flow chamber analysis.
Results : Cell treatment with LPS and S1P significantly enhanced the expression of ICAM-1 and E-selectin, as well as leukocyte-endothelial adhesion, compared with the effect of the ligands alone . On the contrary, no cooperation was observed when a TLR2/TLR1 ligand was used. Next, to elucidate which signaling cascades and S1P receptor subtype were involved in the TLR4-S1PR cross-talk, we used a pharmacological approach that showed differences between arterial and venous cells. In HUVEC, the cooperative effect was attenuated with ERK and p38 inhibitors, sensitive to pertussis toxin, and not blocked with antagonists of S1P receptor subtypes 2 and 3, while in HAEC, the effect was pertussis toxin-insensitive and significantly reduced by blocking S1PR3.
Conclusions: In summary, data demonstrate that TLR4 and S1P receptors cooperate to increase adhesion molecule expression and leukocyte-endothelial cell adhesion, and the signaling cascades and the S1P receptor involved in the

effect differ in arterial and venous endothelial cells.

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Metabolic endotoxemia induces inflammation by selectively removing nuclear receptors and activating C/EBPdelta

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Subclinical levels of circulating endotoxin are associated with the pathogenesis of diverse human inflammatory diseases, by mildly inducing the expression of pro-inflammatory mediators. In this report, we examined the molecular mechanism responsible for the effect of low dose LPS in macrophages. In contrast to a high dose LPS which activates NFκB and induces robust expression of pro-inflammatory mediators, we observed that low dose LPS fails to activate NFκB. Instead, low dose LPS selectively activates C/EBPδ and removes nuclear repressors including PPARα and RARα, enabling a mild and leaky expression of pro-inflammatory mediators. The effect of low dose LPS requires the interleukin-1 receptor associated kinase 1 (IRAK-1). IRAK-1 interacts with and acts upstream of IKKε in contributing to LPS-mediated induction of C/EBPδ and pro-inflammatory mediators. Mice fed with high fat diet acquire elevated levels of endotoxin as well as pro-inflammatory mediators in an IRAK-1 dependent fashion. Taken together, these data reveal a distinct pathway preferentially utilized by low dose endotoxin in initiating low grade inflammation.

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LPS activation of NF-κB-dependant inflammation is attenuated by amniotic fluid in the developing intestine

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Introduction

Lipolysaccharide is a bacterial ligand that binds to Toll-like receptor 4 (TLR-4) leading to the canonical activation of an NF-κB -dependant pro-inflammatory response. Aberrant TLR-4 signaling has been implicated in the pathogenesis of necrotizing enterocolitis (NEC), the leading cause of death from gastrointestinal disease in newborn infants. We hypothesized that amniotic fluid (AF) would attenuate the LPS induced NF-κB activation and spare the developing intestine from inflammation induced disease.

Methods/Results

Using intestinal epithelial cells (IEC-6), amniotic fluid was shown to significantly prevent the LPS induced translocation of NF-κB subunit p65 into the nucleus, correlating with reduced NF-κB activation. To verify these in vitro results, we used a sophisticated high resolution real time ultra-sound guided injection system to administer LPS and AF into the gastrointestinal tracts of fetal (E17) NF-κB -GFP reporter mice. Whole proximal intestines were harvested freshly and subjected to confocal microscopic imaging and 3D reconstruction to evaluate the degree of NF-κB driven GFP production. LPS injection caused a marked increase in GFP signal, which was significantly reduced when LPS was co-injected with AF. Gene expression analysis confirmed that proximal intestinal pro-inflammatory markers iNOS, IL-6 and IL-1ß were attenuated with AF compared to LPS. Strikingly, despite the increase in pro-inflammatory cytokine expression, TLR-4 expression levels were reduced following LPS administration compared with AF administration. This suggests a mechanism by which the fetal intestine down-regulates TLR-4 to prevent a sustained and potentially detrimental inflammatory response.

Conclusions

Amniotic fluid contains factors capable of attenuating the activation of NF-KB pro-inflammatory response. Identifying these factors may provide a clinically relevant solution to pharmacologically prevent uncontrolled TLR-4 responsiveness following preterm delivery of infants.

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The in vivo Pulmonary Cellular Response to Single Wall Carbon Nanotubes: a disruptive immediate march to Inflammation

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Single wall carbon nanotubes (CNT), administered to rats by tracheal instillation (500micrograms suspended by sonication in 50 microliters sterile surfactant) produces an immediate alarm response characterized by eosinophil transmigration into lung tissue and movement of both eosinophils and mucin-laden macrophages (nanoparticle filled?) to the pleural fluid at 3 and 24 hr. High mobility group box 1 protein (HMGB1) increases in bronchoalveolar lavage (BAL) at 30 minutes and is found in the nanoparticle-treated animals at elevated levels for weeks. In order to determine the receptor profile for this "alarm" protein, animals were intratracheally (i.t.) administered 500 micrograms of single wall carbon nanoparticles (CNT) in 50 microliters rat serum. At 0.5, 3, 24 hr and 1 or 4 weeks following CNT instillation, macrophages were harvested from BALs and type 2 pneumocytes were isolated following elastase incubation of blood-cleared lungs. Both macrophages and pneumocytes were incubated with fluorescein-tagged rat-specific antibodies *vs* three putative receptors for HMGB1: TLR9 (intracellular), TLR4 (surface), or receptor for advanced glycation (RAGE, surface). The HMGB1 was also measured by ELISA in plasma and BAL supernatant.

10,000 - 20,000 cells were counted by a FACS Canto cytometer (BD). Fluorescent antibody binding to cell receptors was summarized as percent of the gated cell group corrected for cell fluorescence following staining with isotype controls for each antibody. All CNT receptor signals were also compared to the procedural control, (cells isolated following intratracheal carrier only) .

TLR9 showed consistent elevated expression that averaged 37.4% in macrophages and 17.4% in pneumocytes regardless of the time elapsed after CNT dosage. TLR4 was elevated on pneumocytes as early as 3 hr, remaining elevated on these cells through 4 weeks, while pulmonary macrophages demonstrated TLR4 elevation only at 1 week. RAGE was expressed briefly in pneumocytes (35.95% vs 5% for macrophages) only at 3 hr. The ligand HMGB1 in BAL decreases at 3 hr when TLR4 and RAGE are highest on cells in the lung milieu. Traced over time (out to 4 wk) the inflammatory cytokine TNFα in BAL correlates significantly with BAL HMGB1, suggesting rapid inflammation following the receptor-ligand pairing.

These data detail the rapidity of lung effects produced by a single 50 µL intratracheal dose of sterile carbon nanoparticles in rat serum (<0.03 EU LPS): an immediate extravasation of eosinophils into lung parenchyma ; movement of nuclear protein HMGB1 into the extracellular fluid; increasing cellular expression of "alarm receptors"; increasing inflammatory cytokines. The interesting 4 week increase in HMGB1 and TNFα (endogenous CNT recycling?) was not reflected by any receptor expression .

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Mutations in NLRP3 and CARD8 increases host macrophage resistance to Mycobacterium tuberculosis infection

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Upon *Mycobacterium tuberculosis* infection macrophages produce a range of proinflammatory cytokines needed to overcome microbe-induced alterations of macrophage function, like phagosomal maturation inhibition. Among the repertoire of cytokines is IL-1β, produced upon recognition of *Mycobacterium tuberculosis* by NLRP3 and formation of the inflammasome. In mice, IL-1β production is essential for controlling the infection, though the need of a functional inflammasome *in vivo* is debated. The role of inflammasome activation and subsequent IL-1β signaling during a *Mycobacterium tuberculsosis* infectionof human macrophages has not been as fully elucidated. In the current study, macrophages obtained from two siblings carrying heterozygous point mutations (M299V) in the NLRP3 gene, leading to over-production of IL-1β and presentation of diffuse inflammatory symptoms, were infected with *Mycobacterium tuberculosis*. Following infection, both patients had higher numbers of mature phagosomes compared to control macrophages and cells from one of the siblings were able to control the infection in a caspase-1-dependent manner. Interestingly, this sibling also carries a truncating mutation in the gene encoding for CARD8, which is thought to be a negative regulator of NfκB. Furthermore, inhibition of IL-1β signaling by a receptor antagonist (anakinra) increased intracellular growth of *Mycobacterium tuberculosis* in macrophages from healthy donors. Taken together, these results indicate that an active inflammasome and subsequent IL-1β signaling are crucial for control of *Mycobacterium tuberculosis* in human macrophages.

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Monophosphoryl Lipid A's Trif-biased stimulation of Tlr4 impairs Nlrp3 inflammasome assembly and activation

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Tlr4 plays a significant role in induction of immune responses to both pathogen-associated and endogenously released damage-associated molecular patterns (PAMPs and DAMPs respectively) via induction of the pro-inflammatory cytokine IL-1b. Tlr4 activation by PAMPs and DAMPs has been shown to contribute to the pathogenesis of many IL-1b mediated inflammatory conditions and diseases. Tlr4-dependent IL-1b production involves a priming step involving de novo synthesis of proIL-1b and Nlrp3 and a secondary stimulus, usually in the form of exogenous ATP, for inflammasome assembly and activation of caspase-1. Despite clinical relevance, the contributions of Tlr4's two main signaling adapters, Trif and MyD88, to these processes remain surprisingly undefined. Additionally, a detoxified structural variant of Tlr4's canonical PAMP LPS, named monophosphoryl Lipid A (MLA), is an attractive candidate for inclusion in preconditioning regimens for IL-1b-mediated inflammatory events. MLA is also effective at boosting immune responses when included in vaccine adjuvant preparations; however the primary cause of MLA's reduced toxicity has yet to be reconciled with several hypotheses. We previously characterized MLA as a Trif-biased agonist of Tlr4, and contribute weak activation of the pro-inflammatory MyD88 pathway to be responsible for reduced toxicity compared to LPS. Our results have not yet been reconciled with the finding that MLA does not support

production of mature IL-1b, which could lead to the appearance of Trif-bias by loss of MyD88 signaling potentiation via autocrine activation of IL-1RI. We now report that Trif-biased Tlr4 signaling is the cause and not an effect of MLA's IL-1b loss via a novel mechanism. While Trif-biased signaling efficiently primed murine myeloid dendritic cells at the level of proIL-1b, MLA-primed cells are unresponsive to ATP for inflammasome activation because the critical regulator Nlrp3, whose induction we now characterize as being MyD88-dependent, is not induced.

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Chemically modified NOD-ligands suitable for biological investigations

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The nucleotide binding oligomerization domains-proteins (NOD-proteins) NOD1 and NOD2 play an important role in innate immunity as sensors of components derived from bacterial peptidoglycan (PGN). These proteins are assigned to the NLR-family whose members share a tripartite domain structure of which the LRR domains of NOD1 and NOD2 are thought to interact with bacterial PGN part structures as an initial step of the induction of pro-inflammatory processes. Although the pathogen-associated molecular patterns (PAMPs) of NOD1 and NOD2 have been identified during the last years, the molecular mechanism of the interaction between the NOD proteins and these PAMPs is still unknown. Besides that, less information is available about the intracellular uptake and distribution of NOD-activating PGN and its part structures.

In order to investigate the molecular mechanism of the interaction between the NODs and PGN in more detail, we isolated and chemically modified selected PGN part structures. Particularly, we focused on the (UDP-)muramyl tripeptides [(UDP-)MTPs] from both Gram-positive (Lys-type) and Gram-negative (*m*-DAP-type) bacteria. We considered the ε -amino function of lysine and *meso*-diaminopimelic acid, respectively, to be most promising for chemical modifications. The advantage of this position, with regard to NOD2, is that the minimal recognition motif – the muramyl dipeptide (MDP) – remains exposable for the receptor and unmodified. We introduced a low molecular weight fluorescent marker (7-nitrobenzo-2-oxa-1,3-diazol; NBD) for cell uptake studies. Another approach was the biotinylation of the (UDP-)MTPs to make these PAMPs suitable for binding studies (e.g. Surface Plasmon Resonance measurements).

This presentation will focus on the question, how the chemical modifications (labelling) are influencing the potential of the respective (UDP-)MTPs to trigger an inflammatory response in different *in vitro*-cell systems and – in comparison with commercially available labeled muropeptides – how suitable these modified compounds are for further biological investigations.

This project is funded by the cluster of excellence "Inflammation at Interfaces" (Borstel, Kiel, Lübeck) being part of the integrated research network (IRN-G) "NOD-like receptors – Archetypal Sentinals of Barrier Function".

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There is more to the Solute Carrier family 11 member 1 protein, (SLC11A1): than cytokine regulation

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The Solute Carrier family 11 member 1 protein, (SLC11A1); functions to flux metal cations across the phagolysosome membrane. SLC11A1 links infections, autoimmunity and cancers and is implicated in the susceptibility to these diseases in humans. Our hypothesis is that expression of SLC11A1 due in part to transcriptional regulation causes ionic perturbations to determine cell fate: in accordance with the notion that depletion of cytosol of ions antagonizes cell survival and promotes cell death. MCSF derived macrophages were stimulated with purified TLR-2 agonist : Pam3CSK4. Responsiveness to treatment was determined by comparing microarray gene expression profiles for *Slc11a1*$^{-/-}$ and Slc11a1$^{+/+}$ BMDMs using the Illumina mouse WG 6 V 2.0 beadchip. *Slc11a1*$^{-/-}$ BMDMs were globally refractory to treatment and were bioactive at basal levels, by contrast, Slc11a1$^{+/+}$ were responsive to treatment and were quiescent at basal levels. In human cells, we used SLC11A1 specific RNAi to demonstrate that SLC11A1 influences IL-1 beta release which involves a Pyrin-Inflammasome Complex. In addition, based on our *Slc11a1* microarray gene expression profiling analysis, we show that Slc11a1 regulates p62 sequestosome-1 at basal levels. p62 gene expression was higher for *Slc11a1*$^{-/-}$ BMDMs, Similarly, in *Slc11a1*$^{-/-}$ macrophages, and at basal levels, expression of alternatively activated macrophage markers (M2), tumor associated macrophage markers (TAM), endoplasmic reticulum stress inducers, regulatory T cell inducers, AIM2 and DNA damage response activators were higher. We propose that IL-1beta released whether in exosomes or secreted vesicles should interact with its receptor on adjacent cells, activates the NF-KB pathway for p62 interactions with polyubiquitinated TRAF6. Interactions with speckle or aggresome dwelling p62 would facilitate cell death or survival. Now we know that all of these processes are regulated by SLC11A1 at least at the transcription level. Challenge of Zinc repleted BMDC with BCG caused reduction of DC cell numbers which was accompanied by significant higher hydrogen peroxide levels in protein lysates. These data implies that SLC11A1 influences cell fate desicions and suggests a broader role for SLC11A1 than earlier known.

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Sphingosine-1-Phosphate regulates Human Lymphocyte Functioning via Sphingosine Kinase-1 Activation and Autocrine Signalling

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Sphingolipids represent an important class of bioactive membrane lipids that play a major role in regulating key cellular functions such as activation, cell cycle regulation and migration. The sphingolipid Sphingosine-1-phosphate (S1P) is an important regulator of lymphocyte functioning. Previous studies revealed the effect of S1P acting via the S1P-type 1 receptor (S1P1) on the migration of lymphocytes. However, downstream signalling routes in lymphocyte function following S1P1 activation remain to be elucidated. Therefore, we investigated the role of a self-enhancing autocrine signalling loop acting via S1P1, Sphingosine Kinase1 (SPHK1) and the ATP-binding cassette transporter C1 (ABC-C1) mediated S1P export, on lymphocyte proliferation, migration and activation in vitro. Isolated human lymphocytes were treated with S1P or the specific $S1P_1$ agonist SEW-2871 with or without inhibition of the S1P1, SPHK1 or ABC-C1. S1P induced a concentration dependent increase in proliferation and

chemotaxis of lymphocytes. This was mediated via S1P1as its effect was mimicked by SEW-2871 and could be suppressed by treatment with a specific S1P1antagonist (W123). Furthermore, inhibition of either the kinase (SPHK1) or transport out of the cells (ABC-C1) reduced proliferation, which strongly suggests the existence of a feed-forward loop between S1P1, SPHK1 and the ABC-C1 transporter. Thus, this study shows that S1P activates both proliferation and migration of human lymphocytes. Further, we reveal the presence of a feed-forward loop that acts via activation of SPHK1 following stimulation of S1P1, in which the ABC-C1 mediated S1P transport is of crucial importance. These results underline the importance of $S1P_1$ signalling in lymphocyte functioning, which is not restricted to migration only. Specifically targeting one of these components of the S1P system might be beneficial in treatment of immune-related pathologies such as autoimmune diseases or graft rejection, enabling targeted immunomodulation and reducing possible aspecific side effects. Research support: Groningen University Institute for Drug Exploration (GUIDE)

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Interaction of the Crohn 's Disease Risk Genes, ATG16L1 and NOD2, in an Autophagy-Dependent, Anti-Bacterial Pathway

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Crohn's disease (CD) is a chronic inflammatory bowel disease thought to arise from inappropriate immune responses to bacteria in genetically susceptible individuals. The identification of numerous genes that confer disease susceptibility indicates that this complex disease might arise from alterations in several genes with related anti-bacterial functions. We examined the functional interaction between the CD risk genes *autophagy-related 16-like protein 1* (*ATG16L1*) and *nucleotide-binding oligomerization domain 2* (*NOD2*) to identify an autophagy-dependent, anti-bacterial pathway that is altered by disease-associated variants. Nod2 signaling and autophagy activation in response to the Nod2 ligand, muramyl dipeptide (MDP), was assessed in human epithelial cell lines and primary human macrophages and dendritic cells from healthy individuals genotyped for *ATG16L1* and *NOD2* risk alleles. MDP stimulation of epithelial cells, macrophages, and dendritic cells activated autophagy and NF- κ B and MAPK signaling; it also enhanced killing of the intracellular pathogen, *Salmonella typhimurium*. These responses depended on ATG16L1 and Nod2 expression and were impaired by CD-associated *NOD2* variants. Nod2-dependent signaling was not impaired in cells with the *ATG16L1* T300A genotype, which is associated with CD. However, the *ATG16L1* T300A variant blocked the increase in MDP-mediated killing of *Salmonella* only in epithelial cell lines and not primary macrophages or dendritic cells. Our findings demonstrate that *ATG16L1* and *NOD2* are components of an autophagy-mediated, anti-bacterial pathway that is altered in a cell- and function-specific manner by CD-associated mutations. This work supported by grants from the Crohn's & Colitis Foundation of America and the National Institutes of Health (R01DK082437 and K23DK068112). In addition, these studies were also supported in part by the NIH National Center for Research Resources, CTSA 1UL1RR024989 and the generosity of Nancy & Gerald Goldberg as well as Kenneth & Jennifer Rainin.

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The Inflammasome Response in Term and Preterm Neonates

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One of the human body's most vital functions lies in its ability to fight off invasions from microbes. Immune cells constitute specialized and extremely sophisticated cellular machineries whose goal is to recognize invading micro-organisms and orchestrate a protective response to keep us healthy. However, during the early stages of life, the immune system lies in a state of immaturity and undergoes many changes in order to reach its developed state. This phenomenon can be studied in preterm neonates. Preterm neonates (<29 weeks of gestation) represent a very high-risk population in terms of morbidity from infections. Neonates depend on the innate immune defenses by families of pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) and NOD-like receptors (NLRs) to fight against infections, as the adaptive immune system is not fully developed.
Our study objective was to investigate the inflammasome and TLR response in preterm neonates. When comparing ELISA readings of preterm neonate samples to healthy adults, we and others have reported a decreased ability of preterm infants to secrete the active IL1β cytokine. Although the molecular level at which these developmental differences arise is unknown, intracellular fluorescent stainings of stimulated mononuclear cells have shown that this deficiency arises from the preterms' inability to process proIL1β into the bioactive and secreted IL1β. This suggests that the inflammasome response, the protein multimer that allows for the activation of proIL1β, is deficient in preterms.

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Polymyxin B Enhances IL-1â Secretion in Mononuclear Cells Stimulated with Burkholderia LPS and ATP

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Pathogen-associated molecular patterns and endogenous danger signals, such as bacterial lipopolysaccharide (LPS) and ATP, respectively, trigger the assembly of NOD-like receptors into inflammasome complexes, which control the maturation and secretion of proinflammatory cytokines such as interleukin-1beta (IL-1β). The *Burkholderia cepacia* complex (BCC) is a group of closely-related bacterial species that cause serious opportunistic infections in individuals with cystic fibrosis (CF). BCC LPS is a potent stimulator of innate immune responses, however its ability to induce IL-1β secretion through inflammasome activation has not yet been evaluated. Furthermore, it has been recently noted that polymyxin B (PMB), an antibiotic belonging to a class commonly used to treat infections in CF patients, can enhance IL-1β production. In this study, we evaluated IL-1β secretion from healthy donor peripheral blood mononuclear cells (PBMCs) primed with *B. cenocepacia, B. multivorans,* and *B. vietnamiensis* purifiedLPS, and further stimulated with ATP. PBMCs stimulated with BCC LPS alone secreted minimal amounts of IL-1β, yet normal levels of IL-6, another pro-inflammatory cytokine. ATP increased IL-1β production in a dose-dependent manner. PMB enhanced LPS-mediated IL-1β secretion in the presence of a sub-maximal concentration of

ATP, also a dose-dependent observation. Lactate dehydrogenase cytotoxicity assays revealed no significant cell death, except with the highest concentration of PMB used alongside all other stimuli. These data suggest that PMB can enhance the inflammasome response to BCC LPS, a phenomenon that may contribute to the hyper-inflammatory phenotype of CF *in vivo*. We are currently repeating the experiments with colistin (polymyxin E). This research is supported by CIHR.

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The Innate Immune System and the Clearance of Apoptotic Cells

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Apoptosis, programmed cell death, is used by multicellular organisms to remove cells that are in excess, damaged or diseased. Activation of the apoptosis programme generates "eat me" signals on the surface of the apoptotic cell that mediate recognition and clearance by the innate immune system. CD14, a pattern recognition receptor expressed on macrophages, is widely known for its ability to recognise the pathogen-associated molecular pattern lipopolysaccharide (LPS) and promote inflammation. However, CD14 has also been shown to mediate binding and removal of apoptotic cells in a process that is anti-inflammatory suggesting CD14 is capable of producing two distinct, ligand-dependent macrophage responses. Whilst the molecular basis for this dichotomy has yet to be defined it is clear that CD14 is a point of interest on the macrophage surface where we may study ligand-specific responses of macrophages. Our work seeks to define the molecular mechanisms underlying the involvement of CD14 in the non-inflammatory clearance of apoptotic cells. Here we used three different differentiation strategies to generate macrophages from the monocytic cell line THP-1. The resultant macrophage models were characterised to assess the expression and function of CD14 within each model system. Whilst each macrophage model shows increased levels of surface CD14 expression, our results demonstrate significant differences in the various models' abilities to respond to LPS and clear apoptotic cells in a CD14-dependent manner. TLR4 levels correlated positively with LPS responsiveness but not CD14-dependent apoptotic cell clearance or anti-inflammatory responses to apoptotic cells. These observations suggest CD14-dependent apoptotic cell clearance is not dependent on TLR4. Taken together our data support the notion that the CD14 ligand-dependent responses to LPS and apoptotic cells derive from changes at the macrophage surface. The nature and composition of the CD14-co-receptor complex for LPS and apoptotic cell binding and responses is the subject of further study.

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Allelic mRNA expression imbalance in Inflammasome related genes

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Multiprotein complexes, termed inflammasomes are assembled in a stimulus dependent manner for the activation of caspase-1 and ultimate release of the endogenous pyrogen, interleukin-1beta and interferon inducing factor, IL-18. Because IL-1beta and IL-18 are central to the innate host response, dysregulated inflammasome functioning is associated with numerous diseases and disorders including autoinflammatory diseases, autoimmunity and leukemias. Therefore, naturally occurring genetic variations within any of the components of the inflammasome have potential to modulate the activation and assemblage of this complex. One important variation that , our knowledge, has not yet being systematically investigated in the context of inflammasome function is the possibility that regulatory polymorphisms in specific inflammasome genes may result in altered mRNA expression , which is detectable as an allelic mRNA expression imbalance in subjects heterozygous for a marker SNP in the transcribed exonic region of the genes. This so

called cis acting regulatory polymorphisms are thought to account for a main portion of genetic variability in humans.
To search for these cis acting polymorphisms, we selected 6 genes of particular importance to inflammasome fucntion namely: NLRP1, NLRP3, PYCRD, MEFV, CASP1, and CASP12. We used the public databases to identify TagSNPs in exonic regions that have hjgh frequency of heterpzygosity within these 6 inflammasome genes. We used a single base extension technique (SNapdhot, Applied Biosystems) to measure allele mRNA expression ratios in primary human blood monocytes and MCSF stimulated macrophages in the presence or absence of LPS from heterozygous individuals. Allele mRNA expression was compared to the corresponding genomic sllele ratios measured in the same subjects. A relative deficit in one allele compared to the alternate allele suggests a cis acting regulatory element within the gene locus of interest. Using this approach we predict that specific inflammasome genes will be shown to be regulated at least in part by cis acting genetic elements. We show that similar genotypes were expressed differently. Out of 20 human healthy volunteers 4 heterozygoyes wxhibit monolallelic expression of Pyrin at exon 2 SNPID rs 224225. This we found to be due in part to promoter DNA hypermethylation. In addition, this genomic region is known to be excised from full lenght trabscript during or following gene transcription. For those monoallelic expressions (CASP1 rs539595 in THP-1 cells) that are not found to be due to promoter region DNA hypermethylation, extracellular stimulus such as LPS is able to restore the heterozygous state. We therefore propose and demonstrate for the first time the multiple interacting affects of genetics, epigenetics and exogenous gactors on a biological process such as Inflammaosme Complex activity. This finding has profound implications for the interpretations of genetic linkage and associations studies.

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Inhibition of the Inflammasome Activation: a Survival Strategy of Coxiella burnetii?
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Coxiella burnetii is an intracellular bacterium that causes a severe pneumonia called Q fever in humans. The infection occurs mainly by inhalation of contaminated aerosol and primary target cells are alveolar macrophages. Although *C. burnetii* is known tosubvert several host cell functions, an effective immune response may be able to trigger host resistance. It was previously demonstrated that macrophages from A/J mice strain, which harbors a mutant *Naip5* allele, are more susceptible to *C. burnetii* infection. Since Naip5 is a Nod-like receptor known to trigger caspase-1 activation, we investigated the role of caspase-1 and inflammasomes in recognition and restriction of *C. burnetii* infection in macrophages.
By using a strategy of consecutive backcrosses, we generated mice harboring a restrictive C57BL/6-derived *Lgn1* locus, which includes *Naip5*,in the A/J genetic background. We initially verified that the susceptibility of alveolar, peritoneal and bone-marrow derived macrophages was not correlated to the presence of a restrictive *Lgn1* locus. In agreement, macrophages from *Naip5-/-* mice restricted bacterial infection similarly to C57BL/6 macrophages. We further investigated if Naip5 signaling pathways were activated in response to *C. burnetii* and found that *C. burnetii* induced *irf1* expression independently of Naip5. We also found that macrophages neither failed to activate caspase-1 nor to secrete IL-1β. Moreover, macrophages from *caspase-1-/-* mice effectively restricted bacterial multiplication. Collectively, these data show that neither *Naip5* nor caspase-1 account for restriction of *Coxiella* multiplication. Since we found that caspase-1 was not activated in response to infection, we investigated if *C.*

burnetii was able to inhibit caspase-1 activation. Interestingly, the bacteria inhibited the inflammasome activation via certain stimulus. In conclusion, we demonstrate that, in contrast to other intracellular pathogens, *C. burnetii* not only bypasses NLRs-dependent inflammasome activation, but also is capable of inhibiting caspase-1 activation.
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Inflammasome-Independent Restriction Of Legionella Pneumophila Infection In Macrophages And In Vivo

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Legionella pneumophila is a Gram-negative, facultative intracellular bacterium causative agent of a severe pneumonia called Legionaries' disease. Whereas *L. pneumophila* effectively multiply in human macrophages, murine macrophages restrict bacterial replication in a process dependent on Naip5, Nlrc4 and caspase-1. Thus, the Nlrc4 and Naip5-mediated growth restriction do not support the use of knockout mice in the C57BL/6 genetic background to evaluate the immunity against *L. pneumophila*. The demonstration that bacterial flagellin (FlaA) triggers the Nlrc4-inflammasome support the use of *flaA* mutant *Legionella* to evaluate the host requirements for restriction of *L. pneumophila* infection. **Objective:** To use selected knockout mice in the C57BL/6 background in combination to flagellin-mutant *L. pneumophila (flaA$^-$)* to assess the mechanisms by which the immune system triggers resistance to infection. **Methods and Results**: Mice deficient for cytokines (IFN-γ , IL-6, IL-10, IL-12, IL-17, IL-18, IL-23, TNF receptor) immune cells (CD4 KO, CD8 KO, B KO) or receptors/intracellular signaling (TLR2, TLR9, MyD88, Nod1, Nod2 and Rip2) were infected by *flaA$^-$* and the CFU in the lungs were estimated. In addition, macrophages were obtained from some of these mice and used to evaluate the replication *flaA$^-$* ex vivo. Generally, we found that in response to *L. pneumophila* infection, the macrophage resistance directly correlates with the susceptibility in vivo. Furthermore, we found that the components of the innate immune, but not adaptive immune system are key for restriction of *L. pneumophila* infection. **Conclusion**: Macrophages are key cells for restriction of *L. pneumophila* infection and an effective innate immune response is key for host resistance in a murine model of Legionnaires' disease. **Financial Support**: FAPESP, CNPq, TDR/WHO and PEW.

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Determining the role of IL-1β in Pyoderma Gangrenosum

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Pyoderma gangrenosum (PG) is a rare non-infectious neutrophilic dermatosis, characterized by the formation of sterile abscesses following mild injury (pathergy). PG appears to be an autoinflammatory disease but the pathophysiology is very poorly understood. The aim of these studies was to determine the molecular determinants of disease in a patient with PG. Most PG patients respond well to steroids but currently there is no targeted therapy. Though other auto-inflammatory conditions have been explained at a molecular level, few studies have examined molecular abnormalities specific to PG.
In this study, we investigated a patient with severe PG characterized by multiple episodes of extensive sterile abscess formation after trauma or no obvious provocation and severe inflammatory bowel disease with perforation. All episodes have responded promptly to oral prednisone therapy. We have identified markedly elevated secretion of the pro-inflammatory cytokine interleukin (IL)-1β from the patient's peripheral blood mononuclear cells compared to control cells following stimulation with lipopolysaccharide (LPS) or peptidoglycan and ATP (a signature of auto-inflammation). IL-

1β production was 5 fold greater than control cells after stimulation with LPS as low as 1 ng/ml with ATP. However, protein levels of the I L-1β precursor, pro-IL-1β, and the IL-1ß converting enzyme precursor, pro-caspase-1, were not different from those in control cells upon stimulation. Consistent with effects of IL-1ß on T cell differentiation, $CD4^+$ T cells from the patient produced enhanced levels of IL-17, but not IFN- □, compared to controls. We are currently exploring mechanistic basis of the primary inflammatory dysregulation in this patient and two others with PG. These data suggest that patients with PG have dysregulated innate immunity leading to uncontrolled inflammation. Should a primary defect in control of IL-1ß production be identified, therapy with specific inhibitors (such as anakinra, an IL-1 receptor antagonist) will be considered. This research is supported by the Rare Disease Foundation and the Natural Sciences and Engineering Research Council of Canada.

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Caspase-1 but not IL-18 or IL-1 is Liver Protective after Hemorrhagic Shock in Mice

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Activation of the inflammasome by endogenous danger signals produced during ischemia/reperfusion or hemorrhagic shock leads to maturation of caspase-1, which cleaves proinflammatory cytokines (IL-1β, IL-18) and causes their subsequent release. We have previously shown that caspase1-/- mice had increased liver damage and plasma IL-6 after hemorrhagic shock and bilateral femur fracture compared with C57BL/6 (WT) mice. In this study we investigated whether this effect is dependent on IL-1β, IL18 or caspase-1 itself.
Methods: Caspase1-/-, IL-1R-/-, IL-18R-/- and WT mice underwent sham surgery (femoral artery cannulation only) or HS (1.5h hemorrhagic shock) + 0h, 1.5h, 4.5h or 24h resuscitation (n=3-4/experimental group). Whole-liver lysates were collected and immunoblotted for cleaved (activated) caspase-1. Liver enzymes (ALT, AST) were also measured. Results shown are mean +/- SEM. Data analyzed by Student's t-test, $p<0.05$ considered significant.
Results: Caspase1 was activated (cleaved) in a time-dependent manner in the liver of WT mice, but not the caspase1-/- mice, suggesting inflammasome activation. IL-18 levels were significantly elevated after hemorrhagic shock in the WT mice, but not in caspase1-/- mice. But the levels of IL-1β were comparable between sham and shock group. Caspase1-/- mice had significantly increased AST and ALT levels at 4.5h after shock than the WT mice (ALT: 4824±1831vs 720±146, $p<0.05$) suggesting more liver damage. However, liver damage was similar in IL-1R-/- and IL-18R-/- mice compared with WT mice [ALT: 335±66 (IL-1R-/-) vs 418±121 (IL-18R-/-) vs 567±158 (WT)], suggesting the protective effect of caspase-1 is not mediated by IL-1 or IL-18.
Conclusion: Caspase1 is protective in the liver after hemorrhagic shock independent of IL-1 or IL-18, through a mechanism that has yet to be identified. Caspase1 has multiple cleavage targets that may be important in hepatoprotection. Elucidating the protective effect of caspase-1 in the liver may help understand the regulation of inflammation after trauma and hemorrhage.

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Pathogen-recognition blood proteins – solitary and social combatant(s)

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Specificity in pathogen-recognition is requisite to effective innate immune response. However, the molecular basis of how a protein recognizes

simple structural motifs of pathogen-associated molecular patterns (PAMPs) displayed on the microbial surface, to achieve such precision in self-nonself recognition, remains unclear. We show that PAMP-recognition receptors (PRRs) in the blood can either act alone (eg. hemoglobin) to counter the pathogen or form an interactome (eg. plasma lectins) to become formidable forces against the invading microbe. During an interactome formation, a leading/principle PRR recognizes and binds PAMPs while it simultaneously recruits protein partners onto the pathogen chemo-landscape. Through protein-protein interaction, the interactome collectively increases its affinity for the PAMP by 1000-fold to overcome the pathogen effectively. Furthermore, the host enlists different combinatorials of PRRs to distinguish different groups of invading microbes. Notably, the clustering of PRRs is potentiated by serine-proteases, with a possibility of specific cleavages and conformational changes to the PRRs. Such a frontline defense strategy has been entrenched for several hundred million years, from invertebrates to humans.

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Sushi antimicrobial peptides attenuate multidrug-resistant gram negative bacteria

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The emergence of multi-drug resistance (MDR) gram negative bacteria (GNB) like *Acinetobacter baumannii, Pseudomonas aeruginosa and Helicobacter pylori* is of growing concern. The use of lipopolysaccharide (LPS) binding cationic antimicrobial peptides (AMPs) that induce detergent-like lysis of microbes presents a promising solution against such MDR pathogens. By targeting conserved LPS of GNB membranes, resistance to AMPs could be avoided. Sushi peptides, derived from the LPS binding domains of Factor C (serine protease) isolated from amoebocytes of the horseshoe crab, *Carcinoscorpius rotundicauda* have been shown to possess high affinity for the lipid A moiety of LPS and exhibit prominent bactericidal action. Upon the initial electrostatic association of peptides to GNB membrane targets, Sushi peptides are hypothesized to bacteriolyse in three stages: (1) disruption of outer membrane, (2) permeabilization of inner membrane and (3) disintegration of both membranes. In this study, the antimicrobial efficacy of Sushi peptides were investigated against clinical isolates of MDR *A. baumannii*, *P. aeruginosa* and *H. pylori*. Various bactericidal assays were applied to examine the AMP-bacteria interactions, and characterize the mechanism of action by these peptides. Bioinformatics tools were used in order to relate chemical and physical properties of these AMPs. Finally, these peptides were analyzed for their ability to attenuate LPS-host interactions and were shown to be specific against GNB. (The study is supported by Ministry of Education Grant No. T208B3107)

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Tumor-induced myelopoiesis improves survival to polymicrobial sepsis

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Objective: The mechanism by which tumor-induced adaptive immune suppression occurs has been ascribed to multiple cell populations, including the expansion of $CD11b^{+}Gr\text{-}1^{+}$ myeloid-derived suppressor cells (MDSCs). As many investigators have attributed MDSCs to deleterious effects on host immunity, we examined the role of MDSCs in survival to polymicrobial sepsis during tumor growth. ***Methods:*** Six to eight week old Balb/C mice were injected subcutaneously with either 7×10^3 4T1 or 66C4 mammary carcinoma cells. 4T1 and 66C4 bearing mice with similar primary tumor burden and non-tumor bearing (NT) mice were subjected to cecal ligation and puncture (CLP) and followed for survival. In a second experiment, $Gr\text{-}1^{+}$ splenocytes were harvested and enriched from 4T1 and NT mice and

adoptively transferred into naïve mice before CLP.
Results: We demonstrate that 4T1-bearing hosts, surprisingly, have significantly improved outcome to sepsis when tumor masses are less than 5 % of body weight, compared to either NT or mice bearing 66C4, a clone of 4T1 that does not cause an expansion of MDSCs ($p<0.001$). Improved outcome was associated with increased recruitment of peritoneal macrophages ($F4/80^+$) and neutrophils ($Ly6G^+$) in 4T1-bearing animals following sepsis ($p <0.001$). We also demonstrate that Gr-1^+ cells isolated from 4T1-bearing mice have increased ROS species production in response to PMA stimulation compared to NT animals ($p <0.001$). Additionally, adoptive transfer of Gr-1^+ cells from 4T1-bearing hosts into naive animals was also able to provide protection to CLP compared to animals that were adoptively transferred with Gr-1^+ cells from NT mice ($p <0.05$).
Conclusion: This data suggests that in the setting of MDSC expansion, the myelopoietic response induced by 4T1 tumor growth may actually be protective to polymicrobial sepsis.
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Effects of exposure to fungal cell agents
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Background. Microorganisms are important causative agents for pulmonary inflammatory disease. The effects of the Gram-negative bacteria derived lipopolysaccharide (LPS, endotoxin) on the immune system have been extensively explored but less information is available on agents in fungi. **Aims.** To investigate the effects of exposure to fungal cell agents *in vivo* and *in vitro*. **Material and methods.** Two groups of normal subjects without respiratory disease or symptoms were recruited. In the first group (n=20) peripheral blood monocytes (PBMC) were isolated from venous blood samples and challenged with particulate and soluble β -glucan (Curdlan) and chitin. Measurements were made of TNF- α , interleukin (IL) -6, IL-10, and IL-12. In the second group (n=14) , home exposure to moulds was measured as the amount of airborne β -glucan and blood cell samples were taken.
Results. In the PBMC preparations the secretion of TNF α , IL-6, IL-10 and IL-12 increased strongly after challenge with particulate β -glucan (all $p<0.001$) and less so after with soluble β -gucan and chitin. There was a significant relationship between the amount of β -glucan in bed mattress dust and the percentage of lymphocytes in the blood ($p=0.006$) and an inverse relation to leukocytes ($p = 0.008$). There was no relation with eosinophils or total IgE.
Comments. The results suggest that components of the fungal cell may initiate an acute inflammation as well as a depression of the immune response. This agrees with previous findings using animal models, where the final outcome of the reaction after exposure to β -glucan was related to the dose level. The present data demonstrate that β -glucan and chitin induce reactions in the innate immune system that could be related to the risk of disease caused

by fungi such as asthma, hypersensitivity penumonitis, and sarcoidosis.

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Effects of Avian TREM-A1 Activation on Heterophjil Functional Actvities

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A novel class of innate receptors called the triggering receptors expressed on myeloid cells (TREM) has been discovered and shown to be involved in innate inflammatory responses. The TREM family has been found in the chicken genome and consists of one activating gene (TREM-A1) and two inhibitory genes (TREM-B1 and TREM-B2). However, to date, there have been no reports on the effects of activating the TREM molecules on the functional activity of the primary avian polymorphonuclear cell, the heterophil. To characterize the activation of avian heterophils, we evaluated the effect of receptor ligation on heterophil effector functions. A specific agonistic antibody was generated against the peptide sequence of chicken TREM-A1 38-51aa (YNPRQQRWREKSWC). To study TREM-A1 mediated activation, purified peripheral blood heterophils were incubated with various concentrations of the anti-TEM-A1 Ab or control Ab against an irrelevant Ag. Activation via TREM-A1 induces a rapid degranulation and a dramatic up-regulation in gene expression of the pro-inflammatory cytokines, IL-1β and IL-6, and the inflammatory chemokines, CXCLi2 and CCLi2. However, we found no direct TREM-A1 stimulation of the heterophil oxidative burst. Like mammalian TREM, avian TREM-A1 ligation synergizes with the activation of Toll-like receptor (TLR) ligands LPS and flagellin. In addition, the synergistic activity of TLR and TREM-A1 resulted in the increased production of an oxidative burst. Taken together, these results suggest, unlike in mammalian neutrophils, TREM-A1 engagement activates a differential functional activation of avian heterophils, but like mammalian neutrophils, acts in synergy with TLR agonists. These results provide evidence of the function of TREM-A1 in heterophil biology and avian innate immunity.

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Pharmacological Inhibition of the MD2-TLR4 Complex by Eritoran Reduces Acute Inflammatory Response and Mortality in Experimental Pneumococcal Pneumonia

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TLR4 is the primary receptor for bacterial lipopolysaccharide (LPS), but the same receptor also recognizes host-mediated ligands and other microbial ligands including the pneumococcal exotoxin known as pneumolysin. However, the role of TLR4 in complex Gram-positive infection models is unclear. Eritoran (E5564) is a synthetic analogue of the lipid A from the nontoxic gram-negative bacteria *Rhodobacter sphaeroides*, and has been shown to be a potent inhibitor of MD2-TLR4 signaling in a variety of in vitro and in vivo models, including a human endotoxemia challenge. Eritoran is currently in clinical trials for the treatment of severe sepsis, including sepsis due to pneumonia. *S. pneumoniae* remains the most common cause of severe community-acquired pneumonia, and a frequent etiologic agent of severe sepsis and septic shock. The impact of MD2-TLR4 signal inhibition by eritoran was investigated in vitro and the murine pneumococcal pneumonia model

with or without concomitant antimicrobial therapy (moxifloxacin). Eritoran, tested over a wide range of concentrations, had no consistent effects on murine peritoneal macrophages from either LPS-sensitive (C3H/HeN) or LPS-resistant (C3H/HeJ) mice stimulated by pneumococcal culture supernatants. Thus, TLR4 is not directly activated by pathogen-associated molecular patterns (PAMPS) from *S. pneumonia.*, and eritoran does not directly inhibit cellular activation by these PAMPS. Eritoran had no adverse effect on established pneumococcal pneumonia outcomes in C3H/HeN mice and when combined with moxifloxacin significantly reduced inflammatory markers and improved survival ($p<0.05$). Given the specificity of action of eritoran at TLR4, the results indicate that eritoran reduced the inflammatory response to *S. pneumonia*, likely via inhibition of TLR4 more "downstream" of initial immune cellular activation by the infecting organism.

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Involvement of TLR2 and TLR4 in Cell Responses to Rickettsia Akari

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TLRs are primary sensors of microbial pathogens that activate innate immune cells, as well as initiate and orchestrate adaptive immune responses. However, the role of TLRs in rickettsiae recognition and cell activation remains poorly understood. In this study, we examined the involvement of TLR2 and TLR4 in recognition of *Rickettsia akari*, a causative agent of rickettsialpox. Transfection-based complementation of TLR2/4-negative HEK293T cells with human TLR2 or TLR4 co-expressed with CD14 and MD-2 enabled IκB-α degradation, NF-κB reporter activation, and IL-8 expression in response to heat-killed (HK) *R. akari*. The presence of the R753Q TLR2 or D299G TLR4 polymorphisms significantly impaired the capacities of the respective TLRs to signal HK *R. akari*–mediated NF-κB reporter activation in HEK293 transfectants. Blocking antibodies against TLR2 or TLR4 markedly inhibited TNF-α release from human monocytes stimulated with heat-killed *R. akari*, while TNF-α secretion elicited by infection with live *R. akari* was marginally affected only upon blocking of both TLR2 and TLR4. Both live and heat-killed *R. akari* exerted phosphorylation of IRAK1 and p38 MAPK in 293/TLR4/MD-2 or 293/TLR2 stable cell lines, whereas only live bacteria elicited these responses in TLR2/4-negative HEK293T cells. These data demonstrate that HK *R. akari* triggers cell activation via TLR2 or TLR4, and suggest utilization of additional TLRs and/or NOD-like receptors by live *R. akari*.

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IL-10RB Expression Level but not E47K Genotype Correlates with Cell Responsiveness to IFN-l and IL-22

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IL-10RB is a common receptor subunit required for the function of IFN- λ and IL-22 that affect viral replication and liver inflammation. In the single-nucleotide polymorphisms studies of various populations, E allele of IL-10RB (47K/E) is associated with clearance of Hepatitis B virus (HBV) infection. However, the association of IL-10RB genotypes with hepatocyte-responsiveness to IFN- λ or IL-22

has not been studied yet. By using hepatoma cells over-expressing either IL-10RB E47 or K47 , we investigated the HBV replication and chemotherapeutic agent-induced cell death in response to IFN- λ and IL-22. The suppressive effect of IFN- λ and IL-22 on HBV replication and chemotherapeutic agent-induced cell death correlated with surface expression levels of IL-10RB but not with IL-10RB genotypes. These results suggest that IL-10R K47E genotype may not affect the hepatocyte responsiveness to IL-22 and IFN- λ and other mechanisms for the association of IL-10RB K47 with chronic HBV infection remain to be discovered.

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The Human Cathelicidin LL-37 Preferentially Promotes Apoptosis of Infected Airway Epithelium

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Cationic host defence peptides are key, evolutionarily conserved components of the innate immune system. The human cathelicidin LL-37 is an important cationic host defence peptide upregulated in infection and inflammation, including in the human lung, and has been shown to enhance the pulmonary clearance of the opportunistic pathogen *Pseudomonas aeruginosa in vivo* by as yet undefined mechanisms. In addition to direct microbicidal potential, LL-37 can modulate inflammation and immune mechanisms in host defence against infection, including the capacity to modulate cell death pathways. We demonstrate that at physiologically relevant concentrations of LL-37, this peptide preferentially promoted the apoptosis of infected airway epithelium, with enhanced LL-37-induced mitochondrial membrane depolarisation and release of cytochrome *c*, and activation of caspases -9 and -3, which only occurred in the presence of both peptide and bacteria, but not with either stimulus alone. This synergistic induction of apoptosis in infected cells was caspase-dependent, contrasting with the caspase-independent cell death induced by supra-physiological levels of peptide alone. We demonstrate that the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* required specific bacteria-epithelial cell interaction with whole, live bacteria, and bacterial invasion of the epithelial cell. We propose that LL-37-mediated apoptosis of infected, compromised airway epithelial cells might represent a novel inflammomodulatory role for this peptide in innate host defence, promoting clearance of respiratory pathogens.

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TLRs 2 and 9 Cooperate in the Development of Hypersensitivity Pneumonitis

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Hypersensitivity pneumonitis (HP) is an interstitial lung disease caused by repeated inhalation of environmental antigens. The disease is characterized by alveolitis, granuloma formation, and in some patients, fibrosis. Previous studies in our lab revealed that neutrophils are recruited into the lung via a MyD88-dependent pathway; once in the lung neutrophils regulate IFNγ production which affects granuloma formation and disease severity. The goal of this project was to identify the MyD88-dependent pattern recognition receptors (PRRs) that are responsible for neutrophil recruitment and subsequently granuloma formation during HP. Initial studies indicated that individually, TLRs 2 and 9 contributed to MyD88-dependent neutrophil recruitment; to determine the extent to which they cooperate in the development of hypersensitivity pneumonitis we generated

TLR2/9$^{-/-}$ mice and examined their response in the *S. rectivirgula* murine model of HP. C57BL/6 and TLR2/9$^{-/-}$ mice were intranasally exposed to *S. rectivirgula* one time and neutrophil recruitment measured. TLR2/9$^{-/-}$ mice developed less severe alveolitis compared to WT mice although it was increased above unexposed mice. Flow cytometric analysis of the bronchoalveolar lavage fluid (BALF) revealed a reduction in the % of neutrophils and an increase in the % of activated NK cells and γδT cells in the TLR2/9$^{-/-}$ mice compared to WT mice. The reduction in neutrophils was associated with a reduction in the expression of mRNA for the neutrophil chemokines MIP-2 and KC, and cytokines TNF- α, IL-6, IFNγ and IL-17 in the TLR2/9$^{-/-}$ mice compared to WT mice. ELISA analysis of cell culture supernatants from WT and TLR2/9$^{-/-}$ cells stimulated *in vitro* with *S. rectivirgula* demonstrated that TLRs 2 and 9 contribute to MIP-2 and KC production but not TNF production. To determine the effect of TLRs 2 and 9 on granuloma formation, WT and TLR2/9$^{-/-}$ mice were exposed to *S. rectivirgula* for 3 weeks and granuloma formation in the lungs analyzed by H&E staining. The results demonstrated that TLR2/9$^{-/-}$ mice had less granuloma formation compared to the WT mice following exposure. All together, these results suggest that TLRs 2 and 9 cooperate in neutrophil recruitment through the production of neutrophil chemokines and play an integral role in the outcome of HP. This work was supported by the National Institutes of Health (HL084172).

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Crucial roles of LPS from Neisseria meningitidis and IL10 in altering the TF activity in human monocytes and their monocyte-derived microparticles

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Background : *Neisseria meningitidis* (Nm) causes epidemic meningitis and lethal sepsis with coagulopathy. Bacterial cell wall molecules, particularly lipopolysaccharides (LPS), elicit pathogenic responses including expression of tissue factor (TF). TF may be modulated by tissue factor pathway inhibitor (TFPI), phosphatidylserine (PS) and interleukin 10 (IL10). We have studied the TF-inducing capacity on monocytes (Mo) and microparticles (MPs) using a wild type Nm (wtNm), a mutant lacking LPS (LPS-deficientNm), purified LPS from Nm and E.coli, recombinant IL10 (rhIL10) and antibodies against TF and TFPI. **Methods** Elutriation purified, cryopreserved human Mo (10^6/well) from 3 donors were exposed to 10^6 wtNm, 10^6LPS-deficientNm, 1 ng/mL NmLPS, 1 ng/mL E.coli LPS or vehicle for 4 h, 37^{o}C in presence or absence of 50 ng/mL rhIL10. Mo and supernatants were separated by centrifugation (RT) (50xg, 7 min) and (4500xg, 5 min). Mo were subjected to a clot formation assay (± TF and TFPI antibodies), flowcytometry (TF, PS, annexinV) and RT-qPCR (TF-, alternaltively spliced TF- (asTF) and TFPI- mRNA) . MP-enriched pellets were isolated from supernatants by centrifugation (17,000xg, 30 min) and subjected to a clot formation assay or flowcytometry (CD14, annexinV). **Results** Mo and MPs exposed to LPS showed a high TF-activity compared with LPS-deficient Nm (Mo: 600 mU (wtNm) vs 100 mU (LPS-deficient), MPs: 200 mU (wtNm) vs 25 mU (LPS-deficient)). Anti-TF antibodies and rhIL10 blocked TF-activity, while anti-TFPI abs had no effect. TF expression increased on Mo exposed to wtNm (42%) compared with LPS-deficient Nm (37%), and was reduced by rhIL10 (25% and 21%, respectively), all relative to unexposed Mo. RT-qPCR measurements supported these results. rhIL10 slightly reduced

PS expression on Mo and decreased the number of MPs (CD14+, Annexin V+). **Conclusions** LPS is the principle bacterial component of Nm inducing Mo- and MP-associated TF-activity. These effects are potently attenuated by rhIL10.

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Comparison of Antibodies Against MD-2 and CD14 for Inhibition of LPS or E. coli Bacteria-induced Cytokine Responses in Human Whole Blood

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Background. Sepsis is a major world-wide medical problem with high morbidity and mortality. Gram-negative bacteria are among the most important pathogens of sepsis and their LPS content is regarded to be important for the systemic inflammatory reaction. The CD14/MD-2/TLR4 complex plays a major role in the immune response to LPS. The aim of the present study was to compare the effects of inhibiting MD-2 and CD14 in combination with the complement inhibitor compstatin, on ultrapure LPS- versus whole *E. coli* bacteria-induced responses. *Methods.* Fresh human whole blood was incubated with LPS or whole *E. coli* bacteria in the presence of MD-2 or CD14 neutralizing monoclonal antibodies (mAbs) in the absence or presence of compstatin. Cytokines were measured by a multiplex (n=27) assay and TCC was measured by ELISA. NFκB activity was examined in cells transfected with CD14, MD-2 and/or Toll-like receptors. *Results:* LPS-induced cytokine response was efficiently and equally abolished by MD-2- and CD14-neutralization. In contrast, the response induced by whole *E. coli* bacteria was only modestly reduced by MD-2 neutralization, whereas CD14 neutralization was more efficient. Combination with compstatin enhanced the effect of the MD-2 mAb only slightly, whereas the effect of CD14 mAb was markedly enhanced by compstatin. The cytokine response was virtually abolished by combined CD14 neutralization and compstatin and this combination was the only regimen to abolish the IL-17 response. The MD-2-independent effect observed for CD14, could not be explained by TLR2 signalling. *Conclusion.* Inhibition of CD14 is more efficient than inhibition of MD-2 on whole *E. coli*-induced cytokines, suggesting CD14 to be a better target for intervention in Gram-negative sepsis, in particular when combined with complement inhibition.

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CXCL10 Acts as a DAMP by Inducing MD-2/TLR4-Mediated Nitric Oxide Release

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CXCL10 (also known as IP-10) is a small interferon-inducible chemokine that is rapidly induced and secreted from monocytes, fibroblasts and endothelial cells in response to PAMPs and DAMPs stimuli. CXCL10 plays an important role in modulating immune responses by recruiting T cells and macrophages to the site of inflammation. Elevated CXCL10 levels have been associated with septic and sterile inflammatory diseases. A recent study reported that CXCL10 impairs β cell function in diabetes through TLR4 signaling. The aim of this study was to investigate the interaction of CXCL10 with MD-2/TLR4. Well established murine macrophage RAW264 (TLR4-sufficient) and ScCr (TLR4-deficient) cell lines were stimulated with recombinant murine CXCL10 protein devoid of any LPS contamination. CXCL10 induced a dose-dependent nitric oxide release from RAW264, the TLR4-sufficient macrophages, but not from the TLR4-deficient ScCr macrophages. Boiled CXCL10 (denatured protein) failed to induce nitric oxide release from TLR4-sufficient RAW264 macrophages, which confirmed that CXCL10 protein is responsible for inducing this innate immune response. Similarly, pre-incubation of CXCL10

with polymyxin B did not inhibit nitric oxide release, which ruled out the role of LPS contamination. To confirm the interaction of CXCL10 with MD-2/TLR4, a synthetic lipid A antagonist E5564 was used and resulted in a dramatic inhibition of nitric oxide release. In contrast, recombinant mouse TNF α cytokine induced nitric oxide release from both TLR4-sufficient and TLR4-deficient macrophages. The data indicate that CXCL10 binds to MD-2/TLR4 and induces inflammatory signaling. To verify the signaling pathway induced by CXCL10, RAW264 macrophages were exposed to chemical inhibitors specific for MAP kinases p38 (SB203580), JNK (SP600125) and MEK1/2 (PD98059) pathways prior to CXCL10 stimulation. Compared to RAW264 cells in DMSO, all three inhibitors resulted in a dramatic reduction in nitric oxide release upon stimulation with CXCL10. Taken together, the results suggest that CXCL10 acts as a DAMP and induces innate inflammatory responses via MD-2/TLR4.

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Inhibitory role for IFN-beta in the early immune response to highly pathogenic pulmonary bacteria

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Francisella tularensis , the causative agent of tularemia, is a highly infectious gram negative intracellular bacterium. *F. tularensis* causes an acute disease with a mean time to death of five days following inhalation. One primary feature of tularemia is the ability of *F. tularensis* to suppress the development of innate and adaptive immune responses which are necessary to control and resolve infection. However, the mechanism(s) by which *F. tularensis* modulates host immunity have not been identified. Here we demonstrate a role for IFN- β in the early suppression of inflammation following infection with virulent *F. tularensis* strain SchuS4. Despite exponential replication of SchuS4 in primary human and mouse cells, these cells do not secrete pro-inflammatory cytokines and are refractory to further stimulation with bacterial ligands. Contrasting with the lack of induction of proinflammatory cytokines, primary human dendritic cells (DC) and murine alveolar macrophages rapidly express IFN- β in response to SchuS4 infection. Using IFN- β neutralizing antibodies in human DC and IFN- α/β receptor-deficient airway cells, we show that SchuS4-mediated induction of IFN- β results in the inhibition of specific pro-inflammatory responses following subsequent exposure to unrelated bacterial stimuli. In contrast, IFN- β induced by SchuS4 has no effect on *F. tularensis* infectivity and replication, nor results in an altered rate of cell death. Thus, induction of IFN- β by virulent *F. tularensis* is anti-inflammatory and contributes to the early suppression of innate immunity following infection with SchuS4.

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An alternative conformation of the T cell receptor alpha constant region

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CD8+ and CD4+ T-cells detect peptide antigens, presented by major histocompatibility complex (MHC class I or II) molecules, through alpha-beta T-cell receptors (TcRs) on their cell surface . TcRs are members of the Ig superfamily, the extra cellular regions of the alpha and beta chains each comprising a variable (V) and a constant (C) type domain. The alpha chain constant domain (C ạ) is different from the classical Ig-C1 fold, the potential functional significance of which is unclear. We have determined the ectodomain structure of

an αβ TcR which recognises the auto-antigen MBP. The 2.0Å resolution structure reveals canonical mainchain conformations for the Vα, Vβ, Cβ domains, but the Cα domain exhibits a remarkably different mainchain conformation to those previously reported for TcR crystal structures. The global Ig-C-like fold is maintained, but a piston-like rearrangement between the BC and DE beta turns results in a beta-strand slippage. We propose that this substantial conformational change may represent a signalling intermediate in the TcR upon antigen recognition. Our structure is the first example for the Ig-fold of the increasingly recognised functional concept of "metamorphic proteins".

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Contribution of NOD2 to lung inflammation during Staphylococcus aureus-induced pneumonia

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Staphylococcus aureus is the most commonly found Gram-positive bacterium in patients admitted in intensive care units, causing septicaemia or pneumonia. In this work, we investigated the role of NOD2 in *S. aureus*-induced pneumonia using C57BL/6 mice, which are relatively resistant to *S. aureus* and *Nod2* KO mice on a C57BL/6 background. After intra-nasal instillation of C57BL/6 mice and *Nod2* KO mice with a *S. aureus* strain isolated from a cystic fibrosis patient, or with the laboratory Newman strain, we found that the absence of NOD2 did not modify the mortality, even when the bacteria were used at a concentration as high as 10^9 CFU. In contrast, it affected weight loss and recovery speed: *Nod2* KO mice showed a reduced lung inflammation in comparison to wild-type animals. There was a lower number of CFU in broncho-alveolar lavages on day 2 post-infection in *Nod2* KO mice, but the number of CFU in lung homogenates were similar. There was a lower presence of cytokines (IL-6, IL-1 β , KC, MIP-2, TNF, IFN γ , and IL-10) on day 2 in broncho-alveolar lavage fluids and a reduced recruitment of neutrophils in *Nod2* KO mice. Furthermore, histological analysis of the lungs revealed less severe lesions in *Nod2* KO mice at day 2 and day 7 post-infection. In conclusion, we demonstrated that NOD2 is not a crucial receptor to fight *S. aureus*-induced pneumonia, but that it contributes to the inflammatory response in the lungs. Interestingly, the absence of NOD2 led to a lesser inflammation and was finally beneficial for the animal recovery.

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Peptidoglycan Fragments Released to the Environment; Analysis, Chemical Synthesis and Nod1 Stimulation

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Bacterial cell wall peptidoglycan (PGN) is a potent immunopotentiater and an adjuvant for antibody production. The receptors of PGN were shown as intracellular proteins, Nod1 and Nod2, which recognize diaminopimelic acid (DAP) containing peptide components and muramyldipeptide (MDP) respectively. We have also revealed that DAP-type PGN containing bacteria release Nod1 ligands to the environments [1] . Genetic studies have shown that polymorphisms in human *NOD1* gene significantly associated with susceptibility to several diseases including allergic diseases such as asthma. However, the natural ligands in the the environment, and detailed functions of Nod1 were not really known. We thus separated and determined the human Nod1 (hNod1) ligand in bacterial supernatant (*Escherichia coli* K-12) as GlcNAc-(β1-4)-(anh)MurNAc-Ala- *γ* -Glu-*meso*-DAP [2]. The other PGN fragments were

also determined with introduction of 7-(diethylamino)coumarin-3-carbonyl (DEAC) tag to enhance the ion peaks in the MS analysis [2]. The supernatant of bacillus species, generally inhabiting soil, was also analyzed. The chemical synthesis of the PGN fragments was also achieved , and the observed hNod1 stimulatory activities of the synthesized PGN fragments were consistent with the results of native hNod1 ligands [3]. The results demonstrated the actual molecule to modulate our immune system via hNod1, in the environment.

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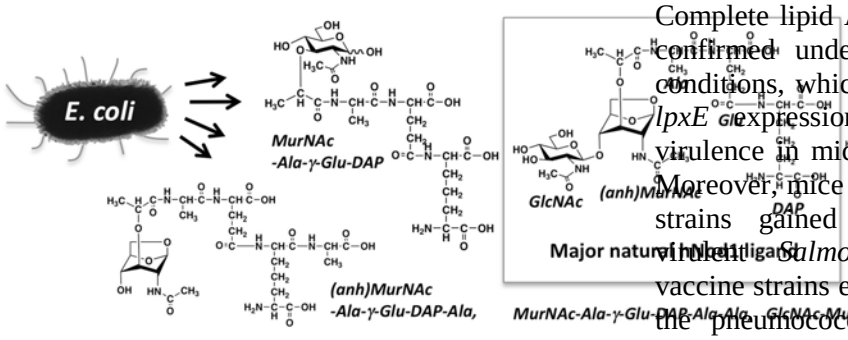

Figure. The major natural human Nod1 (hNod1) ligand and other peptidoglycan fragments released from *Escherichia coli*.

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Live Salmonella Vaccines With Targeted Dephosphorylation of Lipid A

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The development of live, attenuated *Salmonella* vaccines may be facilitated by detoxification of its lipopolysaccharide. Recent characterization of the lipid A 1-phosphatase, LpxE, from *Francisella tularensis* led to construction of recombinant, plasmid-free strains of *Salmonella* that produce predominantly 1-dephosphorylated lipid A, an adjuvant approved for human use. Complete lipid A 1-dephosphorylation was also confirmed under low pH, low Mg^{2+} culture conditions, which induce lipid A modifications. *lpxE* expression in *Salmonella* reduced its virulence in mice by five orders of magnitude. Moreover, mice inoculated with these detoxified strains gained protective immunity against virulent *Salmonella*. Candidate *Salmonella* vaccine strains expressing *lpxE* and synthesizing the pneumococcal surface protein A (PspA) were also confirmed to possess nearly complete lipid A 1-dephosphorylation. After inoculation by the LpxE/PspA strains, mice produced robust levels of anti-PspA antibodies and survived challenge with lethal doses of wild-type *Streptococcus pneumoniae* WU2, validating *Salmonella* synthesizing 1-dephosphorylated lipid A as an antigen delivery system.

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Development of in vitro test method which reflects human originated reactivity for peptideglycan and beta-glucan

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[Objective] As for peptideglycan and beta-glucan, the biological activity is low in comparison with endotoxin, but because there is joint action of endotoxin, necessity of management with the biological formulation is argued. We aimed toward the development of *in vitro* test method which measures the biological activity. This time it examined because of that making use of the human peripheral blood and human peripheral blood originated 28SC cell line. [Materials and Methods] The peptideglycan *Staphylococcus aureus*, *Enterococcus faecalis*, *Lactobacillus acidephilus*, *Saccharomyces cerevisiae* and *Streptomyces spp*., used those of *Micrococcus luteus* origin. It used things such as CM-Curdlan, Zymosan, Laminarin, Sizofiran, Lentinan and *Euglena gracilis* origin as beta-glucan. The measurement reagent and the beta-glucan unique reagent which use the silkworm body fluids were used to the fixed quantity. Biological activity appraised the production of IL-6 by the human peripheral blood and human peripheral blood originated 28SC cell line at ELISA method by measuring. Adding Japanese standard endotoxin, it measured the measurement of joint action between endotoxin and peptideglycan, beta-glucan. [Results and Discussion] Serially diluted peptideglycan and beta-glucan were spiked to human peripheral blood or human peripheral blood originated 28SC cell line. IL-6 production activitys were measured. Dose dependent IL-6 productions were detected. As a result, the peptideglycan which is added and the IL-6 production which depends on the quantity of beta-gulcan were recognized, that production quantity differed considerably depending upon type. In addition, with little dose of peptideglycan and beta-glucan of part which do not show IL-6 production, the IL-6 production reinforcement with endotoxin was shown.

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Studying Intestinal Mononuclear Phagocytes in Tissue and Microflora Context

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Mononuclear phagocytes form a body-wide cellular network devoted to homeostasis, as well as the innate and adaptive immune defense. Mononuclear phagocyte involvement in complex multi-cellular phenomena, such as tissue-remodeling, wound healing, immunity and autoimmunity calls for their study in the intact organism. However, suitable experimental systems to investigate these myeloid linage-derived cells, including blood monocytes and the multiple subsets of tissue-resident macrophages (MF's) and dendritic cells (DCs), *in situ* have long been missing. To study mononuclear phagocyte origins and functions in physiological context, we have developed a combination of *in vivo* cell imaging, ablation and transfer strategies. These approaches enabled us in the past to define organ-specific mononuclear phagocyte origins, as well as the differential requirement of MF's, DCs and DC subsets for T cell immunity.

Here we will report on our recent progress in the study of mononuclear phagocytes in the intestinal *lamina propria*. *Lamina propria* mononuclear phagocytes are believed to be pivotal for robust responses to pathogens and tolerance maintenance towards the commensal microflora. However, their activities in the physiological gut context and functional organization remain poorly understood. The combination of conditional cell ablation and precursor-mediated reconstitution allowed us to establish that the two reported main CD11b- and CD11b+ mononuclear phagocyte subsets have distinct origins and functions. CD11b- DCs arise without monocytic intermediates from MF /DC precursors (MDPs) through a Flt3L-mediated pathway. In contrast, CD11b+ MFs are derived from grafted Ly6C+ monocytes and undergo massive clonal expansion in the *lamina propria*. Mice reconstituted exclusively with monocyte-derived CD11b+ MFs when challenged in an innate colitis model (DSS) develop severe intestinal inflammation driven by graft-derived

TNFalpha-secreting CD11b+MFs. Our results thus highlight the critical importance of the CD11b+/ CD11b- mononuclear phagocyte balance for robust gut homeostasis and establish distinct proinflammatory and regulatory functions of mononuclear phagocytes in *in vivo* context.

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ADAM17 Induction involves Different Cellular Signals during Neutrophil Activation and Induced Apoptosis

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A leukocyte effector activity of mounting significance in the regulation of inflammation is a proteolytic process referred to as ectodomain shedding. This process directs the cleavage of cell surface proteins, regulating the density of receptors and adhesion molecules on leukocytes, and the formation of various soluble agonists and antagonists of inflammation. A key role in ectodomain shedding is played by ADAM17, a membrane-associated metalloprotease. It is known that the induction of ADAM17 enzymatic activity can be acutely regulated posttranscriptionally by cellular signaling events upon cell activation. Of interest, when neutrophils undergoing apoptosis, ADAM17 activity is induced as well; however, the molecular mechanisms involved in the induction of ADAM17 during programmed cell death are unclear at this time. In this study, we investigated the signal events involved in ADAM17 activation upon induced neutrophil apoptosis. We found that a selective inhibitor of caspase 8 (Z-IETD-fmk), but not of caspase 3 (Ac-DMQD-cho), blocked Fas -induced ADAM17 activation; whereas the caspase inhibitors did not block the induction of ADAM17 activity upon neutrophil activation. It is well described that ADAM17 induction upon neutrophil activation involves the p38 and ERK MAP kinases. Though inhibitors of p38 and ERK MAP kinases(SB203580 and U0126, respectively), efficiently impaired ADAM17 induction upon neutrophil action (e.g. fMLP, TNFα , LPS), they did not significantly reduce ADAM17 activity upon Fas-induced neutrophil apoptosis. These data indicate that upon induced neutrophil apoptosis, ADAM17 induction is caspase-dependent , but p38 and ERK MAP kinases-independent, which is the converse of that involved in ADAM17 induction upon overt neutrophil activation. These findings suggest that ADAM17 activity may be an important effector activity by neutrophils during their programmed cell death, and its induction occurs by distinct cellular signals than during neutrophil activation.

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Role of leukocyte ADAM17 in regulating inflammation during E. coli-mediated peritonitis

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Inflammation is the body's initial response to infection, which can be harmful when excessive, as exemplified in sepsis inflammatory syndromes. Ectodomain shedding directs both instantaneous and prolonged alterations in the activity of various cytokines, cytokine receptors, and adhesion molecules, and ADAM17 is a key membrane metalloprotease involved in this process. However, very little is currently known about the *in vivo* function of ADAM17 in regulating inflammation during infection. In this study, we generated *Adam17* gene targeted mice in which only the leukocytes lacked functional ADAM17, and then examined its role in the inflammatory and host responses during *E. coli*-mediated peritoneal sepsis. Conditional

ADAM17 knock-out mice showed increased survival and bacterial clearance during *E.coli*-mediated peritoneal sepsis, which was associated with a reduction in systemic proinflammatory cytokine levels and bacterial burden. Of interest is that we observed a very rapid yet transitory infiltration of neutrophils into the peritoneal cavity of conditional ADAM17 knockout mice when compared with control mice. We are currently examining the mechanisms underlying the enhanced host response in conditional ADAM17 knockout mice, including the molecular processes involved in rapid recruitment of neutrophils. Overall, our study provides the first direct evidence of the instrumental *in vivo* role of leukocyte ADAM17 in modulating inflammation and host resistance during Gram-negative bacterial infection.

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Porphyromonas gingivalis Inhibits RANKL-induced Osteoclastogenesis through down-regulation of NFATc1

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Inflammatory osteolytic diseases including periodontitis and rheumatoid arthritis are usually associated with bacterial infections. However, the precise mechanisms by which bacteria induce bone loss still remain unclear. Evidence exists that Toll-like receptor (TLR) signaling regulates both inflammation and bone metabolisms and that receptor activator NF-kappaB ligand (RANKL) and its receptor RANK are the key regulators for bone remodeling and for the activation of osteoclasts. We have shown recently that the periodontal pathogen *Porphyromonas gingivalis* inhibits RANKL-induced osteoclast formation from uncommitted osteoclast precursor cells through TLR2/MyD88 pathways. The purpose of this study was to investigate the cellular mechanisms by which *P. gingivalis* inhibits RANKL-induced ostoeclastogenesis. Our results showed that *P. gingivalis* inhibits RANKL-mediated induction and activation of NFATc1, the master transcription factor for osteoclastogenesis. This inhibition was not mediated through down-regulation of RANK or the induction of IFN-beta. However, *P. gingivalis* suppressed RANKL-induced sustained activation of JNK and NF-kappaB. Increased levels of ERK, p38 and Akt activation were induced by *P. gingivalis*. In addition, RANKL-induced activation of CREB/ATF-1 and expression of c-Fos were inhibited by this pathogen. Taken together, these results suggest that *P. gingivalis* inhibits RANKL-induced NFATc1 and osteoclastogenesis by regulating multiple intracellular signaling pathways. Supported by a University of Alabama at Birmingham Center for Metabolic Bone Disease pilot project award and NIH grant DE14215.

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Crosstalk between TLR, CD40 and BCR in regulating B cell responses to antigens of Francisella tularensis

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B cells are important antigen-presenting cells (APCs) and like other APCs, Toll-like receptor (TLR) signaling in B cells has the potential to modulate humoral and cell mediated immune responses. Our studies with a bivalent subunit vaccine against *Francisella tularensis* infection have demonstrated the effectiveness of the DnaK and Tul4 antigens from *F. tularensis* in conferring protection against this pathogen. I n the present study, we investigated the role of TLR signaling in DnaK or Tul4 mediated activation of B cells and its crosstalk with CD40 and BCR. Tul4 strongly activated B cells to produce IL-6 and IL-10 and to upregulate the expression of MHC-II and costimulatory molecules, while DnaK induced ten-fold lower cytokine levels. Activation of B cells by Tul4 or DnaK required TLR2 or TLR4 signaling, respectively. Costimulation with CD40 significantly increased the level of cytokines induced by DnaK as well as Tul4. However,

activation of B cells via TLR and BCR appeared to lower the level of IL-6 and IL-10 induced by TLR/CD40 signaling. Multiplex cytokine analysis following DnaK and Tul4 stimulation revealed a differential crosstalk between TLR and CD40 or BCR signaling for B cell responses. CD40 signaling revealed an additive effect on Tul4 or DnaK induction of IL-6 and IL-10, whereas BCR signaling enhanced Tul4 or DnaK mediated production of MIP-1 α, MIP-1 □ and IL-7. Noteworthy, combined CD40/BCR signaling stimulated B cells to produce a wide array of proteins including IL-1 α, IL-6, IL-7, IL-9, TNF- α, IL-10, MIP-1 α, MIP-1 □, G-CSF and RANTES suggesting the involvement of different signaling pathways and a crosstalk between TLR/CD40/BCR signaling. Understanding this interplay should be an integral part in the design of potential vaccines capable of inducing optimal protective immune responses. Supported by NIH grant DE09081.

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Novel Peptide Antagonist Therapy for Superantigen- and Endotoxin-induced Lethal Shock

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Superantigens (SAg) are bacterial exotoxins that cause a massive cellular immune response leading to lethal shock. SAgs directly bind to the outer surface of MHC-II molecules on antigen presenting cells and the outer face of the variable β chain of the T cell receptor, bypassing conventional antigen presentation. This leads to over 20 % of the T cells to undergo polyclonal expansion with the release of proinflammatory cytokines, causing a T helper 1 cytokine storm and subsequent shock. Recently, peptides derived from T-cell costimulatory receptors were found to protect D-galactosamine (D-gal)-sensitized mice from lethal challenge with the SAg, staphylococcal enterotoxin B (SEB). In the absence of D-gal, outbred (CD1), BALB/c and C3H mice are resistant up to 200ug of SEB; therefore in preparation for use of a more physiological model of SEB toxicity, SEB/LPS synergistic lethality, we first assessed the protective efficacy of such a peptide against LPS or SEB alone. In D-gal sensitized mice, the peptide protected 66 % of the mice (8/13 treated vs 2/13 untreated mice, $p<0.0044$) from lethal challenge with LPS and 100 % of mice (5/5 treated vs 0/5 untreated) from lethal SEB challenge. To determine the effect of the peptide against live infections, mice were injected with either SAg-producing *S. pyogenes* or *E. coli* treated with a subtherapeutic dose of antibiotics to better demonstrate antagonism. The peptide protected 80 % of mice from lethal challenge with *S. pyogenes* (8/10 treated vs 2/10 untreated mice, $p<0.0034$) and 100 % of mice (15/15 treated vs 0/10 untreated mice) from *E. coli* challenge. To better understand how the peptide protected against LPS lethality, TLR4-, MD2-, and CD14- transfected HEK cells were stimulated *in vitro* with LPS in the presence of peptide and found to have significantly less luciferase (NFκB) expression than those stimulated without peptide, indicating that the peptide may block LPS signaling through TLR4. However, activation of NFκB by Pam3Cys or flagellin in a TLR2- or TLR5-dependent manner was not blocked by the peptide. We also found no antagonist activity of the peptide in HEK cells that were transfected with a plasmid carrying CD4 (ectodomain)/TIR and

constitutively expressing NFκB. This suggests the requirement of either the TLR4 surface receptor or MD2 in mediating peptide action. We conclude that the peptide may be useful in protecting against SAg- and/or LPS-mediated lethal shock or against active infection.

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Bovine colostrum reduces gut inflammatory lesions and circulating interleukin-1beta and interleukin-8 in preterm pigs susceptible to necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is a serious gastrointestinal disease in preterm infants. Bovine colostrum is rich in bioactive factors which could protect the preterm infant from detrimental inflammatory reactions leading to development of NEC. We aimed to investigate the protective effect of colostrum (native (unmodified) as well as sterilized and spray-dried colostrum powder) on the protection of neonatal piglets against NEC following preterm birth and infant formula feeding. The focus was on correlations between pig cytokine induction and NEC development. Preterm caesarean-delivered pigs were fed total parenteral nutrition for 48 hr followed by 2 boluses of infant formula to create a pro-inflammatory state. Subsequently, pigs were divided into four enteral nutrition groups: Reconstituted gamma -irradiated bovine colostrum powder (F-POWDER, n=8); reconstituted gamma -irradiated+pasteurised bovine colostrum powder (F-PASTPOWDER, n=9); gamma -irradiated bovine colostrum (F-COLOS, n=14) or infant formula (FORMULA, n=14). The severity of NEC was evaluated with a pathological NEC score (range 1-6: Six being severe NEC). A five-plex immunoassay was used to measure the concentration of interleukin (IL)-1β, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α in distal small intestinal tissue. NEC severity was higher in FORMULA pigs (2.8) than F-PASTPOWDER pigs (1.5; $p<0.001$) and F-POWDER pigs (1.6; $p<0.01$), whereas F-COLOS pigs only showed a tendency towards lower NEC severity than FORMULA pigs (2.2; $P=0.058$). The concentration of IL-1 β in distal small intestinal tissue was significantly lower in the F-PASTPOWDER group than the FORMULA group ($P<0.01$) with intermediate levels in the two other colostrum groups. A two-fold higher concentration of IL-8 in distal small intestinal tissue was observed in FORMULA pigs compared with pigs in the three colostrum groups. Data for the three other cytokines were below detection limit. Spray-drying and pasteurization of bovine colostrum did not reduce the positive effect on NEC severity of colostrum, and processed bovine colostrum may still act to modulate the immune system in a beneficial direction, reducing IL-1 β and IL-8 production. Therefore, processed bovine colostrum could serve as an anti-inflamatory source of enteral nutrition for the preterm neonate.

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How Does LPS Stimulate B cells in vivo?

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LPS is the prototype T-independent type I antigen, yet how it activates B cells *in vivo* has been a controversial issue in immunology for over 3 decades. Is the 'one nonspecific signal' delivered directly to B cells by LPS sufficient to induce antibody production, or is a second signal required from accessory cells? And why is LPS, a potent B cell mitogen *in vitro*, such a poor stimulus to antibody production *in vivo*? To address these questions, we first transfused LPS-responsive ($Tlr4^{+/+}$) or non-responsive ($Tlr4^{-/-}$) B cells into responsive or nonresponsive mice. Transient up-regulation of the early activation marker, CD69, could be induced on transfused $Tlr4^{-/-}$ B cells by injecting LPS into $Tlr4^{+/+}$ mice, demonstrating indirect activation of non-responsive B cells by

responsive accessory (non-B) cells *in vivo.* This early activation of *Tlr4*$^{-/-}$ B cells did not lead to antibody production. In contrast, *Tlr4*$^{+/+}$ B cells did not up-regulate CD69 when they were transfused into LPS-injected *Tlr4*$^{-/-}$ mice yet they produced large amounts of IgM. Antibody production thus required direct LPS-B cell contact and occurred without prior CD69 up-regulation. We next tracked fluorescent and radiolabeled LPS molecules from a subcutaneous injection site to its draining lymph nodes (DLN). Although LPS reached the DLN within minutes of injection, drainage continued for many weeks and most of the LPS was inactivated by acyloxyacyl hydrolase-mediated deacylation before it reached the DLN. Very little LPS was found outside the subcapsular sinus and medulla. Conclusions: productive B cell activation requires prolonged delivery of fully acylated LPS to the DLN and direct LPS-B cell contact. Supported by NIH extramural grant AI18188 to UT Southwestern Medical Center and by the Division of Intramural Research, NIAID, NIH.

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Kill the Bacteria…and Also Their Messengers? The Resolution Phase of Innate Immunity.

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Although several host molecules can modulate the stimulatory activities of bacterial lipopolysaccharides, LPS inactivation *in vivo* requires deacylation by a host lipase, acyloxyacyl hydrolase (AOAH), that removes two of the six fatty acyl chains from lipid A (Nat. Immunol. 6:989, 2005). Whereas wildtype mice recover from endotoxin tolerance (macrophage "reprogramming") within 5 to 10 days, in *Aoah*$^{-/-}$ mice the tolerant state lasts for ≥4 months after *in vivo* LPS exposure and is associated with impaired resistance to bacterial challenge (Cell Host Microbe 4:293, 2008) . Here we report that LPS deacylation is required for recruited monocytes to differentiate into LPS-responsive macrophages. When LPS is injected into the peritoneal cavity of a normal mouse, many of the resident peritoneal macrophages depart and are replaced by blood monocytes, which then differentiate into macrophages. In *Aoah*$^{-/-}$ mice that were primed with intraperitoneal LPS two weeks earlier, the "macrophages" recovered from peritoneal fluid had monocyte-like markers (low SSC, CD11b^{lo}, CD86^{lo}, F4/80^{lo}), retained stimulatory (fully acylated) LPS, and were unable to produce pro-inflammatory mediators when stimulated *ex vivo* with LPS or other microbial products. **Summary:** the prolonged immunosuppression that sometimes follows Gram-negative bacterial infection may be caused by persistently active LPS and/or the host's inability to deacylate it. Microbial agonists may retard monocyte differentiation into tissue macrophages.

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What Happens to LPS in Animal Tissues?

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The extraordinary potency and pathological relevance of Gram-negative bacterial lipopolysaccharides (LPS) have made them popular experimental agonists, yet little is known about what happens to these stimulatory molecules within animal tissues. We tracked LPS from a subcutaneous inoculation site to the draining lymph nodes (DLN), blood and liver. Within minutes of injection, FITC-LPS was found in DLN. Drainage continued, with kinetics suggesting passive transport, for many weeks. Cell-free LPS moved through the subcapsular sinus and medulla of the DLN, where it could be found on or within lymphatic

endothelial cells and macrophages. In wildtype mice, ≥ 70% of the injected LPS was partially deacylated before it left the footpad and no fully acylated LPS was recovered from the liver; in animals that lacked acyloxyacyl hydrolase, the deacylating enzyme, prolonged drainage of bioactive LPS induced B cell proliferation and boosted polyclonal IgM and IgG3 antibody titers. In this *in vivo* model, LPS egress from the injection site was largely via lymphatics, not the bloodstream, and enzymatic deacylation played a major role in determining its downstream stimulatory potency. Supported by NIH extramural grant AI18188 to UT Southwestern Medical Center and by the Division of Intramural Research, NIAID, NIH.

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Peptidoglycan Recognition Proteins Protect Mice from Inflammatory Bowel Disease by Promoting Normal Gut Flora and Preventing Induction of Interferon-γ

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There are multiple mechanisms of maintaining tolerance in the gut that protect the intestine from excessive inflammatory response to intestinal microorganisms. We report here that all four mammalian Peptidoglycan Recognition Proteins (PGRPs or Pglyrps) protect the host from colitis induced by dextran sulfate sodium (DSS). Pglyrp1$^{-/-}$, Pglyrp2$^{-/-}$, Pglyrp3$^{-/-}$, and Pglyrp4$^{-/-}$ mice are all more sensitive than wild type (WT) mice to DSS induced colitis; they exhibit greater loss of body weight, higher mortality, severe hyperplasia of the lamina propria, loss of epithelial cells, and ulceration in the colon. A genome-wide analysis revealed higher production of interferon- γ and interferon-inducible genes in all PGRP-deficient mice compared to WT mice upon exposure to DSS. Analysis of cell populations in the colon demonstrated an increase in NK cells in PGRP-deficient mice and identified NK cells as the source of interferon- γ . In vivo neutralization of interferon-γ or depletion of NK cells significantly reduced the severity of colitis. Analysis of the gut microflora demonstrated a shift in PGRP-deficient mice to bacteria with higher proinflammatory activity compared to WT microflora. Colonization of germ-free mice with microflora from PGRP-deficient mice or WT mice demonstrated that the gut microflora plays a crucial role in the increased pathology in PGRP-deficient mice. Thus, in WT mice PGRPs protect the colon from early inflammatory response and the loss of barrier function of intestinal epithelium by promoting growth of normal bacterial flora and by preventing increased production of interferon-γ by NK cells in response to injury. This work was supported by USPHS Grants AI028797 and AI073290 from NIH.

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TLR and NLR induced inhibitory molecule CD200 and phagocytic receptor MARCO enhance host immunity, but limit excessive inflammation to protect host from immune pathologies

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Activated macrophages are host-protective, but uncontrolled activation leads to immuno-pathologies. We compared microbe-induced “innate”, Th1 (IFN-gamma) and Th2 (IL-4) cytokine mediated “classical” and “alternative” activation. We identified the scavenger receptor MARCO and inhibitory molecule CD200 as selective markers for innate activation, induced by the gram-negative human pathogen Neisseria meningitidis (NM) and purified PAMPs. Induction of MARCO was dependent on the MyD88 pathway; whereas CD200 induction depended on TLRs-MyD88-NF-κB signalling; as well as NOD2 and NALP3. Furthermore, we identified a unique epigenetic mechanism by which chromatin conformation for these two gene loci was dynamically altered during activation, allowing their expression.

The class-A scavenger receptors MARCO and its related molecule SR-A, are phagocytic PRRs, unable to initiate inflammatory responses by

themselves, yet implicated in various immuno-pathologies. Once induced, both MARCO and SR-A recognised NM and increased its phagocytosis. Furthermore, coligation of SR-A/MARCO along with selected TLRs and NLRs by their shared ligands, showed that SR-A and MARCO attenuated the response by surface-expressing TLR4, while enhancing responses by the intracellular TLR3, NOD2 and NALP3. These data suggest that SR-A/MARCO-mediated rapid ligand internalisation prevented sustained sensing by surface TLRs, while increasing ligand delivery into intracellular compartments, allowing sustained sensing and robust responses by intracellular sensors.
CD200 binds to its inhibitory receptor providing an inhibitory signal to receptor bearing cells. CD200-/- macrophages were hyper-responsive to TLR and NLR agonists. $CD200^{-/-}$ animals were susceptible to meningococcal septicaemia, induced high levels of inflammatory cytokines and recruited increased number of activated leukocytes despite comparable bacterial clearance. Thus CD200 is induced by TLR- NOD2- and NALP3- mediated pathways limiting their function and protecting the host from excessive inflammation.
We conclude that innate activation induces functional markers, which in combination provide enhanced host immunity while limiting an excessive host response, protecting the host from inflammatory pathologies.

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Slr from Streptococcus pyogenes – a surface lipoprotein interacting with type I collagen

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Streptococcus pyogenes is an important human pathogen and surface structures allow it to adhere to, colonize and invade the human host. Proteins containing leucine rich repeats (LRR) have been indentified in mammals, viruses, archaea and several bacterial species. They are mostly involved in protein-protein interaction mediated through the LRR region. The LRRs are typically 20-30 amino acids long and the defining feature of the LRR motif is an 11-residue sequence LxxLxLxxNxL (x being any amino acid). The streptococcal leucine rich (Slr) protein has been recently discovered and is presumed to be a lipoprotein attached to the cell membrane.
There are presently no ligands identified for Slr. Based on our previous knowledge of LRR proteins we hypothesized that Slr could mediate binding to collagen. We could establish that Slr is a membrane attached lipoprotein by electron microscopy and immunoblotting Furthermore, we did *in silico* protein sequence analysis and homology modeling and analyzed the interaction between recombinantly expressed Slr or an isogenic *slr* mutant strain (MB1) with collagen type I. We could confirm that Slr is a membrane attached lipoprotein. We could observe a weaker binding capacity of collagen type I to MB1 as compared to the AP1 wild type strain. *In vitro* experiments with gold-labeled Slr showed multiple binding sites to collagen I, both to the monomeric and the fibrillar structure. Most binding occurred in the overlap region of the collagen I fibril, but further experiments are required to reveal the specific binding sites.

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Differential HIV-1 Endocytosis in MDMs Correlates with the Activation State of the Cells

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HIV-1 usually enters host cells by pH-independent fusion at the plasma membrane. However, in monocyte-derived macrophages (MDMs), viral endocytosis may occur simultaneously, possibly allowing for productive infection. We are currently investigating the role and mechanisms of HIV endocytosis in human MDMs. Using GFP labeled R5-viruses and real time live-cell confocal microscopy, we were able to observe HIV-1 endocytosis and accumulation in acidified compartments stained with Lysotracker Red. Laser scanning confocal immunofluorescence microscopy of fixed cells further showed partial colocalisation of HIV with the endosomal/lysosomal proteins EEA1, CD63 and Lamp-1. Using different drugs (Chlorpromazine, Dimethylamiloride) and a p24-ELISA assay that allows for quantitation of viral fusion and endocytosis, we identified clathrin-mediated entry as the major mechanism of HIV-1 endocytosis in MDMs. Clathrin-mediated endocytosis is gp120 dependent, as blocking it did not have a significant effect on Env-negative HIV-1 internalization. However, clathrin involvement in HIV internalization is variable between different blood donors. Variability may be linked to the state of cellular activation, as cells of the monocytic-macrophage lineage show a high level of plasticity in response to their micro-environment. We are currently investigating the impact of this polarization (M1, M2a) on HIV endocytosis and on its fate inside MDMs. Preliminary results suggest that MDM activation/polarization has an impact on both entry efficiency and the mechanisms used for HIV-1 entry. This work was supported by the CIHR (M.J.T.). L.-A.G. is the recipient of CIHR and FRSQ PhD studentships.

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Protein A from Staphylococcus aureus signals type I interferons through the TLR4 receptor complex

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Staphylococcus aureus is a major human pathogen causing significant morbidity and mortality as a result of pneumonia and skin infections and is increasingly becoming resistant to current antimicrobial therapies. A major virulence factor of *S. aureus* is protein A that is capable of signaling through TNFR1 and EGFR as well as activating the type I interferon response. The type I interferon response is important in the control of invasive pathogens activated through intracellular receptor complexes. A canonical pathway for this activation is the interaction between LPS and TLR4. Having previously established that staphylococci potently activate the type I IFN cascade in the pathogenesis of pneumonia, we now demonstrate that this is accomplished through the interactions of protein A (SpA) and the TLR4 complex. Using dendritic cells isolated from the corresponding null mutant mice, we found that the expected components (TLR4, TRIF, MD2 and CD14) as well as the downstream transcription factor TBK1 were required for induction of IFN-beta and phosphorylation of STAT1 in response to SpA. Endocytosis of SpA was accomplished through its known association with EGFR. In addition, TLR4 appears to be main receptor for SpA proinflammatory signaling as both KC and IL-6 expression were significantly reduced in TLR4 null dendritic cells. In vivo experiments confirmed the ability to SpA to act like LPS, as SpA expression was required to induce sepsis in mice and purified SpA introduced intraperitoneally stimulated cytokine secretion and inflammation. These observations suggest that the *S. aureus* virulence factor protein A activates signaling through the TLR4 receptor complex to activate the type I IFN response.

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Dysregulated Cofilin Phosphorylation in Siblings with Recurrent Infections, PMN Dysfunction, and Cytoskeletal Defects

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Three Qatari, whose parents are first cousins, had frequent severe skin and mucosal ulcerations, recurrent upper and lower respiratory tract infections, and neutropenia. The younger sister had repeated oral infections that resulted in development of oral strictures. A twin sister of the younger child with a similar presentation died at age 3 from a septic event. Three other children were normal. Assessment of functional responses of PMN isolated from the two living sisters identified profound defects in cell spreading, fMLF-induced shape change, and chemotaxis *in vitro*. PMN showed abnormal nuclear morphology in as much as 90% of the cells with herniation of nuclear lobes into plasma membrane-enclosed surface blebs. Electron microscopic examination of isolated cells confirmed abnormal morphology in both PMN and eosinophils. Moreover, herniation of nuclear lobes coincided with retraction of the granules into the central region of the cells, resulting in an agranular region in the periphery of the cell. Confocal microscopy with AlexaFluor546-phalloidin showed that cells with herniated nuclear lobes exhibited increased levels of F-actin compared to normal PMN. Analysis of the cells by flow cytometry showed 4-5-fold increase in the phalloidin staining of the herniated cells.

Two-dimensional gel electrophoresis of PMN lysates identified several cytoskeleton-associated proteins that were differentially expressed in PMN isolated from the sisters compared to PMN from normal subjects. In particular, cofilin, identified on the 2-D gel in two discrete spots, was down-regulated in one spot compared to normal, yet up-regulated in the other spot, suggesting a difference in post-translational modification of cofilin. Though overall cofilin expression was normal, phosphorylation of cofilin at Ser3 was almost completely absent in both sisters compared to an unaffected sib and normal subjects. Disruption of the cofilin pathway in these sisters represents a novel defect in PMN that likely represents an autosomal recessive mutation.

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***Francisella tularensis* Modulates Membrane Trafficking in Human Monocyte-Derived Dendritic Cells**

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Francisella tularensis is the highly infectious bacterium that causes tularemia, and its virulence has raised concern about its potential use as an agent of bioterrorism or biowarfare. Previous work has determined that the organism can escape from the phagosome of professional phagocytes, but its fate in human dendritic cells has not been determined. Using confocal laser scanning microscopy and electron microscopy, we now show that *F. tularensis* enters and replicates within human monocyte-derived dendritic cells (hMDDCs), and that by seven hours post-infection, approximately 70% of bacteria are located in the cytosol. Although *Francisella*-containing phagosomes initially colocalize with late endosome markers, these

markers are shed simultaneously with phagosome escape. The nature of the phagosomal compartment from which the bacterium escapes is incompletely defined. We have found that early phagosome maturation is altered and that cysteine proteases important to antigen processing (cathepsins S and L) are never efficiently recruited to the phagosome. We hypothesize that antigen processing and presentation are impaired by both phagosomal escape and exclusion of cathepsins. Ongoing experiments will assess the effects of these observations upon phagosome maturation and antigen presentation.

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TRIF Induces Proteolytic Degradation of TLR5

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Proteolytic modification of pattern recognition receptors (PRRs) and PRR-associated adaptor molecules is a key cellular event that regulates the receptor function and the receptor-mediated immune and inflammatory signaling. For instance, proteolytic cleavage of TLR9 is required for full activation of TLR9 and caspase-dependent cleavage of TIR domain containing adaptor inducing IFN- β (TRIF) results in attenuated antiviral immunity. So far proteolytic degradation of TLR5 has not been reported. In this study we found that TRIF, an adaptor molecule mediating TLR3 signaling and MyD88-independent signaling of TLR4, plays an inhibitory role in TLR5-elicited responses by inducing proteolytic degradation of TLR5. TRIF overexpression reduced the level of TLR5 protein, not mRNA, in human embryonic kidney (HEK293) and colonic epithelial (NCM460) cells. Moreover, flagellin/TLR5 driven NF κB reporter activity was suppressed by TRIF overexpression, whereas MyD88 overexpression did not alter the activity in NCM460 cells. Other TLRs including TLR3, TLR6, TLR7, TLR8, TLR9 and TLR10 are also degraded by TRIF overexpression with the exception of TLR1, TLR2 and TLR4. Furthermore, TRIF-induced TLR5 protein degradation is completely inhibited in the presence of pan-caspase inhibitor (Z-VAD-FMK), while several specific inhibitors against cathepsin B, reactive oxygen species or ubiquitin-mediated proteasome activity fail to suppress this degradation. Finally, we identified that the C-terminal region of TRIF and the extracellular domain of TLR5 rather than its cytoplasmic TIR domain are required for TRIF-induced TLR5 protein degradation. Collectively, our findings propose a potential inhibitory role of TRIF by showing that TRIF mediates proteolytic degradation of TLR5 and attenuates TLR5 responses. Thus, TRIF plays a key role in regulating TLR5-dependent host-microbial communication in colonic epithelial cells.

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TRIF mediates Toll-like receptor 5-dependent inflammatory signaling in the intestine

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TLR5, specifically recognizing flagellin, is involved in promoting the pathophysiology of inflammatory bowel disease (IBD) by mediating a host-microbial interaction in the gut. TLR5 is abundantly expressed in the gastrointestinal epithelial cells and flagellin response is seen in a subset of lamina propria dendritic cells, $CD11c^{+}CD11b^{+}$, in the small intestine. Unlike TLR4 which mediates both MyD88- and TRIF-dependent signaling, so far a single adaptor molecule MyD88 is known to solely mediate TLR5-induced responses to elicit inflammatory responses. To the best of our knowledge, the involvement of TRIF in TLR5-dependent signaling has not been suggested . Here we found that TRIF physically interacts with TLR5 upon flagellin stimulation in human colonic epithelial cells (NCM460), while TRAM does not bind to TLR5. Silencing TRIF expression in these cells reduced TLR5-induced nuclear factor κB (NF κB) and mitogen-activated protein

kinases (MAPKs), specifically JNK1/2 and ERK1/2 activation and this inhibitory effect was similarly observed in MyD88 silenced cells. Moreover, silencing both TRIF and MyD88 completely inhibited activation of NF κB and MAPKs. Primary intestinal epithelial cells isolated from TRIF knock-out (KO) mice showed significantly reduced flagellin-induced NF κB (p105 and p65), JNK1/2 and ERK1/2 activation compared to control cells from TRIF-wild type (WT) mice. Moreover, TRIF-KO intestinal epithelial cells exhibited substantially reduced inflammatory cytokine (KC, MIP-3 α and IL-6) expression upon flagellin compared to the WT control cells. In an *in vivo* intestinal inflammation model, TRIF-KO mice were resistant to flagellin-mediated exacerbation of colonic inflammation and DSS-induced experimental colitis compared to TRIF-WT mice. In conclusion, in addition to MyD88, TRIF mediates TLR5-dependent responses and, thereby regulates inflammatory responses elicited by flagellin/TLR5 engagement. Our findings suggest an important role of TRIF in regulating host-microbial communication via TLR5 in the gut epithelium.

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The endoglycosidase EndoE of E. faecalis and its possible role as an immunomodulating enzyme

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Many human pathogens have evolved mechanisms to escape the recognition of the immune system or to modulate the responses directed against the pathogen. *Enterococcus faecalis* is one of the most common pathogens involved in nosocomial infections. It can cause bacteremia, infectious endocarditis and urinary tract infections.

A recently identified gene of *E. faecalis*, named *ndoE*, encodes for the novel endoglycosidase EndoE, which contains a hexosaminidase and a chitobiase domain closely related to human enzymes. Besides cleavage of high mannose glycans on human RNase B, EndoE hydrolyzes also the N-linked glycans on IgG. Moreover, the glycans on IgG are important for proper functioning of IgG e.g., binding to Fc receptors as well as activation of complement. Cleavage of glycans can influence the evasion of the innate immune response and therefore contribute to the development of the disease.

The goal of this study is to investigate the possible immunomodulating function of EndoE. Therefore, thirty clinical *E. faecalis* isolates from different sources were investigated for the presence and secretion of EndoE. An ndoE knockout mutant will help to verify the contribution of EndoE as an immunomodulating enzyme to virulence and persistence of *E. faecalis*.

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Induction of Myeloid Cell Interleukin-10 Production in Response to Leishmania Involves Inactivation of Glycogen Synthase Kinase-3β Downstream of Phosphatidylinositol-3 Kinase and Upstream of cAMP Response Element Binding Protein

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The leishmaniases represent a wide spectrum of diseases caused by protozoa of the genus *Leishmania*. Mononuclear phagocytes are the primary target cells for leishmania infection. In the absence of intervening antimicrobial treatment, leishmania disease expression and resolution is determined by the balance of inflammatory and immunosuppressive cytokines produced. One of the most critical determinants

is interleukin-10 (IL-10) which promotes disease progression by virtue of its immunosuppressive properties. Here we investigated the regulation of IL-10 production by macrophages infected with *Leishmania donovani*, an agent of visceral leishmaniasis. Infection of either murine or human macrophages with leishmania brought about selective phosphorylation of serine/threonine protein kinase Akt-2 in a phosphatidylinositol-3 kinase (PI3K) dependent manner. This was linked to phosphorylation (serine-9) and inactivation of glycogen synthase kinase-3β (GSK-3β) as phosphorylation at serine-9 was completely abolished by inhibition of either PI3K or Akt. One of the transcription factors regulated by GSK-3β is the cyclic AMP response element binding protein (CREB). We investigated the impact of leishmania infection on the activating phosphorylation of CREB at serine-133 and CREB DNA binding activity. Infection of macrophages with leishmania induced phosphorylation of CREB and this was associated with enhanced DNA binding activity and the production of IL-10. Like phosphorylation of GSK-3β, phosphorylation of CREB at serine-133, enhanced DNA binding activity of CREB and IL-10 production were all abrogated in cells treated with inhibitors of either PI3K or Akt prior to infection. Finally, disruption of this pathway via over-expression of GSK-3β also blocked activation of CREB and markedly attenuated IL-10 production in response to leishmania infection. These findings establish that the macrophage IL-10 response to leishmania is regulated by a pathway production consisting of PI3K-Akt-GSK3β and CREB.

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Activation of Dectin-1 upon formation of a "phagocytic synapse"

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Innate immune cells must distinguish between direct binding to microbes and detection of components shed from the surface of microbes located at a distance. The pattern recognition receptor Dectin-1, which is expressed by macrophages, dendritic cells and neutrophils, plays a crucial role in anti-fungal defense. Dectin-1 detects β -glucans in fungal cell walls and triggers direct cellular anti-microbial activity, including phagocytosis and production of reactive oxygen species. In contrast to inflammatory responses stimulated upon detection of soluble ligands by other pattern recognition receptors, such as Toll-like receptors (TLRs), these responses are only useful when a cell comes into direct contact with a microbe and must not be spuriously activated by soluble stimuli. In this study we show that despite its ability to bind both soluble and particulate β -glucans, Dectin-1 signaling is only activated by particulate β -glucans, which cluster the receptor in synapse-like structures from which regulatory tyrosine phosphatases CD45 and CD148 are excluded. The “phagocytic synapse” provides a model mechanism by which innate immune receptors can distinguish direct microbial contact from detection of microbes at a distance, thereby initiating direct cellular anti-microbial responses only when they are required.

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ATP-sensitive potassium channels mediate survival during infection

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The clinical syndrome of sepsis is typified by tachypnea and alterations in leukocytes, temperature and heart rate. Organ failure has a cumulative effect on mortality, due predominantly to cardiovascular collapse, and lung and kidney failure. Clinical outcome depends on host genetic factors, pathogen virulence factors and co-morbidities, yet studies that probe these interactions are limited. Assessments of cytokine and pathogen levels have proven to be poor predictors of patient mortality, highlighting a need to develop novel mouse models of sepsis. A screen of ENU-mutagenized mice disclosed a mutation in *Kir6.1* causing profound susceptibility to infection by mouse cytomegalovirus and *Listeria monocytogenes* as well as ~20,000-fold sensitization to LPS, dsRNA, and CpG DNA. The LPS hypersensitivity phenotype was not suppressed by mutations in *Myd88*, *Trif*, *Tnf*, *Tnfrsf1a*, *Ifnb*, *Ifng*, *Nos1*, *Nos2*, *Il6*, *Ncf1* or *Stat1* however a compound mutation in *MyD88* suppressed the hypersensitivity to CpG DNA. Kir6.1 combines with a second protein, SUR2, to form an ATP-sensitive potassium channel (K_{ATP}) that confers cardiovascular stability during responses to infection. Reconstitution of lethally-irradiated wild-type recipients with Kir6.1 mutant bone marrow cells did not confer LPS hypersensitivity, nor did it affect the ability of chimeric mice to respond to *Listeria monocytogenes*, indicating that Kir6.1 acts in a manner extrinsic to the hematopoietic system. A Kir6.1 mutation rendered mice highly susceptible to infection with *Plasmodium berghei*, with death occurring 5 days after infection. No differences in parasitemia were noted between Kir6.1 mutant and wild-type mice. Reconstitution of lethally-irradiated Kir6.1/TLR4^{P712H} mutant mice with wild-type bone marrow cells restored sensitivity to LPS, indicating that hematopoietic cells provide the trigger for cardiovascular collapse in Kir6.1 mutant mice. This mouse model may help to unravel the clinical conundrum of sepsis and understand the morbidity associated with K_{ATP} mutations in humans.

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LPS is the allergy-protective principle of Acinetobacter lwoffii F78

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Exposure to farming environment during early childhood has been identified In numerous epidemiological studies to strongly influence the development of allergic reactions later in life. Moreover, it had been shown that certain bacteria isolated from this environment have allergy-protective properties in mouse allergy models (Debarry et al, JACI 2007). In the present study, we further characterized one of these bacteria, namely the Gram-negative *Acinetobacter lwoffii* F78, with regard to the bacteria-induced signaling and possible mechanisms of allergy protection. The impact of *A. lwoffii* F78 on human monocyte-derived dendritic cells especially with respect to their T helper cell polarization capacity was investigated by ELISA, FACS analysis and real-time PCR experiments as well as confocal microscopy.

We could show that *A. lwoffii* F78 induces a T_H1-polarizing program in human dendritic cells, including the upregulation of T_H1-driving molecules such as ICAM-1, IL-23p19 or CCL5 as well as the induction of IFN- γ release by co-cultured autologous naïve T cells. In addition, TIM-3 expression was induced and the TBet/GATA3 transcription factor expression ratio was shifted towards T_H1. Blocking experiments using anti-TLR2 antibodies and lipopolysaccharide (LPS)/TLR4-antagonists

indicated that the LPS of *A. lwoffii* F78 was the responsible molecule promoting these effects. Moreover, isolated LPS from *A. lwoffii* could fully substitute for whole bacteria regarding all investigated effects of the activation. In addition, we are currently investigating epigenetic control mechanisms for the driving of T_H1 polarization, in particular the effect of different histone deacetylases.
In summary, we found evidence that the allergy-protecting effects of *A. lwoffii* F78 are due to the activation of a T_H1-polarizing program in human dendritic cells, and that the LPS of *A. lwoffii* F78 is responsible for these beneficial effects (supported by DFG, SFB/TR22, project A2).

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Salmonella enterica serovar Typhimurium lipopolysaccharide deacylation enhances its intracellular growth within macrophages
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Modification of lipid A is essential for bacterial adaptation to its host. *Salmonella* Typhimurium LpxR potentially detoxifies lipid A by 3'-*O*-deacylation; however, the involvement of deacylation in its adaptation remains unclear. LpxR-dependent 3'-*O*-deacylation was observed in the stationary phase. When macrophages were infected with stationary phase bacteria, the intracellular growth of the *lpxR*-null strain was lower than that of the wild-type strain. Furthermore, the expression level of iNOS was higher in the cells infected with the *lpxR*-null strain than in the cells infected with the wild-type strain. These results indicate that lipid A 3'-*O*-deacylation is beneficial for intracellular growth.

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Chronic Granulomatous Dsease in Israel: Clinical, Functional and Molecular Sudies of 48 Patients
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Chronic granulomatous disease (CGD) is an innate immunodeficiency due to a genetic defect in one of the NADPH-oxidase components. In the course of 21 years, 48 Israeli CGD patients from 38 families were diagnosed with 22 gene mutations, eleveven of which were new. The CGD patients included 34 males [70.8%] and 14 females [29.2%]; 29 [60.4%] Jews and 19 [39.6%] Arabs. Clinical, functional, and molecular studies were accomplished. Fourteen patients (29.2%) from 13 families had the X-linked subtype with a defect in membrane subunit $gp91^{phox}$ and 34 (70.8%) from 25 families had the autosomal recessive (AR) subtype, 8 with a defect in the membrane subunit $p22^{phox}$, 14 in the cytosolic subunit $p47^{phox}$, and 12 in the cytosolic subunit $p67^{phox}$. Severe clinical expression was found both in the XLR and AR forms, but in general a milder disease was evident in AR-CGD, particularly in patients with p47phox deficiency. Patients with the X-linked subtype had an earlier disease onset (0.6±1 year vs 3.4±2.8 years, respectively, *P*=.004) and lower survival rate (Kaplan-Meier method, mean±SE: 19.4±2.7 years vs 40.3±7.7 years, respectively; *P*=.03 6, log rank test). Despite early and aggressive therapy, a mortality rate of 26% was noted. Given that bone-marrow transplantation was successful in five of seven patients, it is recommended to perform it as early as possible before tissue damage is irreversible.

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Benefits of Heat-Treatment in the Protease Packed Neutrophil: Halting Protein Degradation
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Introduction & Aims
Neutrophils are the main cells of the innate immune system. They contain an array of proteases and reactive oxygen species which aid to control the invasion of bacteria and pathogens but can also result in damage to healthy tissue. The quantity of intracellular proteolytic enzymes makes it a difficult cell to work with due to the risk of degradation of proteins of interest. Here we describe the prevention of protein degradation in the neutrophil using the "Stabilizor T1" device from Denator.
Method
Neutrophils isolates from 4 healthy volunteers were each divided into 3 and subjected to different preparation methods for 2D PAGE; 1) Samples were heat-treated using the Stabilizor T1, 2) Resuspended in NP40 Lysis Buffer, 3) Resuspended in standard 2D lysis buffer (both lysis buffers contained protease inhibitors). All 12 samples were acetone precipitated and 2D PAGE was carried out. Gels were silver stained and image analysis was carried out using Progenesis software. Spots of interest were excised and identified by Mass Spectrometry (MS).
Results
Samples resuspended in standard 2D lysis buffer displayed such an extent of sample degradation they could not be aligned with NP40 and heat-treated samples for image analysis. Heat-treated samples contained proteins (identified by MS) at high molecular weights that were absent from NP40 samples. NP40 samples showed an increase in spot number at lower molecular weights in comparison to heat-treated samples suggesting protein degradation.
Conclusion
Halting protein degradation in the neutrophil is beneficial for all further downstream analysis of neutrophil proteins, particularly for 2D PAGE, the primary method for the detection of isoforms and post-translational modifications. Use of the Stabilizor T1 results in the identification of proteins that may not have previously been detected in a neutrophil isolate due to sample degradation, thus leading to a definitive proteome map of the human neutrophil.
Research Support: Mater Misericordiae University Hospital, Irish Heart Foundation.

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Neutrophil Function and Clinical Correlates of 998 Pediatric and Adult Patients with Recurrent Infections: the Israeli Report
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Objectives: To analyze the prevalence and clinical significance of a variety of phagocytic dysfunctions in patients referred to our laboratory over a 21-year period. **Patients and Methods:** A group of 998 pediatric and adult patients, who had recurrent infections and no known background of immune deficiency or systemic disease, were included in the study. Initial workup included chemotaxis, superoxide production (SOP), and bactericidal activity (BA). Based on test findings, further evaluation, including genetics studies, were conducted. **Results:** The most common clinical manifestations were recurrent skin and subcutaneous (46.6%), lower respiratory tract (22%), and purulent upper respiratory tract (16.6%) infections. Deep-seated abscesses, sepsis and CNS infections were found in 5.5%, 5.3% and 1.9% of patients, respectively. ROC analysis showed that the more the organ systems involved, the greater the chance of phagocytic impairment. Phagocytic dysfunction was found in 33.6% of the patients; defective chemotaxis in 16.6%, SOP in 6%, and BA in 24.5%. A primary phagocytic disorder was identified in 57 patients (5.7%).
Conclusions: The high rate of idiopathic phagocytic dysfunction could be related to deleterious effects of the persistent infection, to

pharmacological influence of therapeutic agents or to other disorders not yet established.

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Potentiation of Epithelial Innate Host Response by Intercellular Communication
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The epithelium efficiently attracts immune cells upon microbial infection despite the low number of pathogens and moderate levels of secreted chemokines per cell. Here we examined whether horizontal intercellular communication between cells may contribute to a coordinated response of the epithelium. *Listeria monocytogenes* infection, transfection, and microinjection of individual cells within a polarized intestinal epithelial cell layer was performed and activation was determined on the single cell level. Surprisingly, chemokine production after *L. monocytogenes* infection was primarily observed in non-infected epithelial cells despite invasion-dependent cell activation. Whereas horizontal communication was independent of gap junction formation, cytokine secretion, ion fluxes, or nitric oxide synthesis, Nox4-dependent oxygen radical formation was required and sufficient to induce indirect epithelial activation. In summary, this study describes epithelial communication in response to innate immune activation significantly contributing to anti-infectious host defence at the very early stage of microbial challenge.
T.D. was supported by a Ph.D. grant from the Hannover Biomedical Research School, Center for Infectious Biology postgraduate training program. C.C. was funded by a Postdoctoral Fellowship from the Humboldt Foundation. M.W.H. was supported by the German Research Foundation (Ho2236/5-3), the German Ministry for Science and Education (BMBF 01KI0752 and 01GU0825) as well as the Collaborative Research Center (SFB 621 and SFB 900).

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The contribution of alternatively activated macrophages in eliciting the hypersusceptibility to post-influenza bacterial pneumonia
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Background: A lethal bacterial pneumonia is a common complication of influenza. The mechanisms responsible for the increased risk of bacterial infection after influenza are not completely known. We developed a model of post-influenza secondary bacterial pneumonia in which we show enhanced lethality during recovery from influenza, when the host has cleared the virus. Unlike classically activated macrophages, alternatively activated macrophages (AA-MΦ) have poor antibacterial activity. Therefore, we explored the potential contribution of AA-MΦ to the hypersusceptibility to the succeeding infection.
Methods: C57BL/6 mice were intranasally (IN) challenged with a 10-fold sublethal dose of H1N1 murine-adapted influenza virus A/PR/08 (PR8) and 14 days later followed by a >100-fold sublethal dose of *Streptococcus pneumoniae* serotype 3 (Sp3, ATCC 6303 strain). Paraffin-embedded lung sections were stained for Mac-3 and YM1. Lung homogenates were examined by real time PCR for genes associated with AA-MΦ.
Results: With sublethal PR8 infection, mice became ill and lost weight, but by day 10, mice recovered and gained weight, and viable virus was not detected from lung or BAL. However, mice that completely recovered from PR8 infection (14 d post-PR8) demonstrated marked mortality with sublethal Sp3: 2 of 17 mice survived among the PR8-recovered *versus* 11 of 11 survivors among PR8-naïve controls (log rank test, $p<0.0001$). The deaths coincided with rapid systemic spread of bacteria within <48 h, as confirmed by colony counts of lungs, liver, spleen, and blood. Upon *ex vivo* stimulation with LPS, CpG, or Pam_3Cys, the pro-inflammatory cytokine responses of spleen homogenates from PR8-recovered mice, in comparison to PR8-naive mice, were blunted.

On immunohistochemical staining of lungs 18 h after bacterial infection, YM-1 positive macrophages were observed in PR8-recovered mice, whereas few were detected in PR8-naïve mice. Furthermore, there was significantly increased expression of arginase-1, FIZZ1, YM1, IL-4, and IL-13 mRNA, all markers of AA-MΦ, in mice recovering from PR8. **Conclusions:** We show host hypersusceptibility to a sublethal bacterial infection during recovery from influenza infection and provide evidence for the presence of AA-MΦ during this time. We propose that AA-MΦ contribute to poor host defenses post-influenza infection.

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Imbalance of T regulatory, Th17, and Tc17 cells in the early stage of Nocardia brasiliensis infection in BALB/c mice

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Mycetoma is a chronic infectious disease produced by fungi or bacteria; in the latter it is called actinomycetoma. One of the causative pathogens is *Nocardia brasiliensis*, which is a gram positive, facultative intracellular bacterium that lives as saprophyte in soil and enters human skin by traumatic inoculation. Nocardia infection induces an inflammatory response with multiple microabscesses and granuloma formation with abundant large cells and mononuclear infiltration, mainly lymphocytes. Lymphocyte subpopulations are being studied to evaluate their role in immunopathogenesis of several infectious diseases. In this study, we evaluated the levels of Treg, Th17 and Tc17, as well as their related cytokine levels during *Nocardia brasiliensis* infection in BALB/c mice. Mice infected with *N. brasiliensis* were sacrificed at 3, 7, 15, 30 and 60 days after infection and levels of Treg, Th17 and Tc17 cells and their related cytokines (IL-10, IL-17A, IL-23, and TGF-β) were measured by flow cytometry and ELISA, respectively. Our results show that Th17 and Tc17 were increased at day 3 ($P<0.0001$ and $P<0.003$), while Treg cells are increased at day 7 infection ($P<0.0001$). However, from day 15onwards, the three subpopulations decrease until they presented the same levels. On the other hand, anti-inflammatory cytokines levels were higher (TGF-β at days 7 and 30, $P<0.01$; IL-10 at day 15, not significant). IL-17A and IL-23 levels were increased gradually during the infection ($P<0.01$ and $P=0.007$, respectively). In conclusion, there is an imbalance of Treg, Th17 and Tc17 in the early stage of *N. brasiliensis* that could favor chronic disease.

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HIV-1 ssRNA Promotes Foam Cell Formation in Human Macrophages

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Introduction Atherosclerosis occurs with increased incidence and accelerated rates in persons infected with HIV, although the mechanism is incompletely understood. Intracellular accumulation of lipids in macrophages leads to characteristic “foamy cell” formation, a hallmark of atherosclerotic lesions. Although antiretroviral medications can influence lipid metabolism, whether HIV infection is associated with altered macrophage lipid trafficking has not been established.

Methods and Results Peripheral blood monocyte-derived macrophages were obtained from consenting healthy adult volunteers (under the auspices of BIDMC IRB) using Ficcol-Hypaque density centrifugation and CD14+ enrichment (magnetic beads), cultured with M-CSF x 5 days at 37^{o}C. Macrophages were in vitro infected with R5 (M-tropic) HIV Bal

isolate x 2 weeks (infection confirmed by release of HIV RNA), and incubated with oxLDL x 24 hrs. Compared to uninfected monocyte-derived macrophages, in vitro HIV infected macrophages demonstrated significant accumulation of intracellular lipid (oil red "O" or Bodipy staining and microscopy). To examine the mechanism, human monocyte-derived macrophages expressed intracellular TLR7 and TLR8 staining (flow cytometry) and released TNF-a (ELISA) in response to TLR7/8 agonist (Imuquimod). Pretreatment of human macrophages with HIV ssRNA x 24 hrs, then incubated with oxLDL resulted in significant accumulation of intracellular lipid.

Conclusions Collectively, these data demonstrate that HIV infection of human macrophages results in intracellular lipid accumulation, independent of antiretroviral medications. Furthermore, HIV ssRNA is sufficient to promote intracellular lipid accumulation in human macrophages. Altered intracellular lipid trafficking in macrophages may promote "foamy cell" formation and contribute to accelerated rate of atherosclerosis in HIV+ persons.

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Antimicrobial Peptide Inhibits Antiviral Inflammatory Responses

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Manipulation of macrophage and dendritic cell function with immunomodulators has therapeutic applications ranging from vaccine adjuvants to cancer immunotherapy. Immunomodulators are recognized by Toll-like receptors (TLRs) and promote the expression of various cytokines, chemokines and inflammatory mediators. However, recent data have shown that under certain conditions, TLR stimulation can be enhanced to a level that induces immunopathology. In psoriasis, damaging levels of interferon-beta production are due to high levels of the c ationic antimicrobial peptide LL37, which binds to DNA and enhances TLR9 signaling. Double stranded RNA (dsRNA) induces type I interferon production through TLR3. Therefore, we asked whether LL37 might similarly enhance TLR3 responses. Mouse macrophages and dendritic cells were stimulated with the synthetic TLR3 ligand poly (I:C) in the presence or absence of human (LL37) or mouse (mCRAMP) antimicrobial peptide. In the presence of antimicrobial peptide, TLR3 signaling was dramatically inhibited. Secretion of TNF- α, upregulation of IL-6 and IL-1 β mRNA and production of nitric oxide were all significantly inhibited. Most importantly, poly (I:C)-induced interferon-beta production was blocked. It is important to note, that this is in direct contrast to the augmentation of TLR9 signaling by antimicrobial peptide where it is proposed that LL37 enhances delivery of DNA to TLR9. Instead we found that antimicrobial peptides and poly (I:C) directly interacted very strongly. We propose a new model where formation of a complex with LL37 blocks TLR3 access to poly (I:C), thereby inhibiting signaling. Our novel findings highlight the importance of using caution when therapeutically delivering nucleic acids. During existing acute or chronic inflammation, where LL37 levels are elevated, the resulting immune response could be dramatically altered.

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Activation of the agr system in S.aureus after ingestion by neutrophils contributes to neutrophil lysis

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The increasing incidence of infections due to community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is a growing clinical concern. One of the predominant CA-MRSA strain types in the

United States is pulse field gel type USA300. The underlying basis for virulence associated with CA-MRSA USA300 in human hosts is not well understood.
The *agr* system in *S.aureus* is a known global regulator of virulence factors in vitro and is associated with increased disease severity in animal models of staphylococcal infection. This study investigated the influence of the *agr* system and one of its downstream virulence factors, α-hemolysin (hla), on the fate of polymorphonuclear neutrophils (PMN) and *S.aureus* ingested by PMN. PMN were fed with the CA-MRSA USA300 strain LAC, the *agr*- mutant, or the *hla*- mutant, at various multiplicity of infection (MOI). The viability of the intracellular bacteria was determined by colony plating, the bacterial expression of RNAIII was analyzed by real-time PCR and PMN lysis over time was measured using the lactate dehydrogenase release assay. We found that only phagocytosed organisms rapidly increased transcription of RNAIII in a time-dependent fashion; extracellular LAC did not exhibit increased RNAIII expression despite having the capacity to respond to autoinducing peptide-enriched culture medium. The survival of the LAC strain was similar to the *agr*- mutant, however greater PMN lysis was observed with the LAC strain relative to the *agr*- mutant at a MOI of 5:1. The *hla* mutant also caused less PMN lysis relative to the LAC strain. CA-MRSA USA300 strain LAC actively and rapidly responded to being ingested by human PMN. The activation of the *agr* system and the subsequent production of α-hemolysin contributed to PMN lysis at MOIs>1. Further investigation into the interplay between CA-MRSA and the host cell in the context of the phagosome may shed light on the contribution of these factors in staphylococcal disease.

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Role of WNT5a signaling in Francisella infection

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Wnt5a, a 43 kDa secreted glycoprotein, has been associated with the receptor Frizzled 5 (Fz5), and shown to initiate a signaling cascade that follows the non-canonical Wnt pathway. Studies have demonstrated the involvement of Wnt5a in processes such as cancer suppression, neurodevelopment and osteoarthritis, but only recently has Wnt5a been implicated in the inflammatory response resulting from infection with intracellular pathogens. The infectious process caused by the intracellular pathogen *Francisella tularensis* is characterized by a severe inflammatory response thought to adversely affect the host. Therefore, we investigated the involvement of Wnt5a in the host's inflammatory response to *F. tularensis* LVS (*Ft*-LVS) infection, and the signaling molecules involved in its regulation. Murine bone marrow-derived macrophages (BMDM) infected with *Ft*-LVS were used in these studies. The role of Toll-like receptors (TLR) in the induction of Wnt5a/Fz5 was assessed using TLR knockout mice. Our results indicate that Wnt5a is involved in the host response to *Ft*-LVS as determined by the induction of Wnt5a and Fz5 following stimulation of BMDM. *Ft*-LVS is an agonist of TLR2/1 and TLR2/6 signaling; however, the induction of Wnt5a was mainly dependent on TLR2/1. Furthermore, following *Ft*-LVS stimulation of BMDM, the MAP kinase ERK and the glycogen synthase kinase 3 (GSK3) played a role in the early and late induction of Wnt5a, respectively, whereas JNK signaling exerted a negative regulatory effect on Wnt5a expression. Wnt5a was implicated in the down-regulation of IL-6 production at early (8 h), but not at later (24 h) times, as observed by increased IL-6 levels following blocking of Wnt5a. These results suggest a regulatory role for Wnt5a in the inflammatory process caused by *Ft*-LVS. The full implication of Wnt5a involvement in the

infectious process will require further investigations. Supported by NIH training grant T32 AI-07051.

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Exposure to Mycobacterium avium biofilm results in inability to ingest and kill bacteria by resting and activated macrophages (Mo)

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Introduction: Mycobacterium avium forms biofilms. Studies in vitro have suggested that M. avium in biofilm is resistant to antibiotics commonly used for treatment of the lung disease in humans. In experimental animals, the ability to form biofilm is associated with efficiency to establish disease. The reason why biofilms are established without host reaction in first place, is currently unknown. We hypothesize that biofilms can resist to the host innate immune defense, and we studied whether macrophages (Mo) and NK cells can affect M.avium biofilm at different stages of evolution.
Methods: MAC 104 and A5 biofilm were established on glass, plastic and polarized human bronchial cells. After 14 days, THP-1 Mo were added to the biofilm. At 4, 24 and 48 h the biofilms were monitored for CFU and scanning EM. In some experiments, Mo were also stimulated with IFN-γ and TNF-α for 24 h before adding to the biofilm. In addition, biofilms were seeded and exposed to NK cells alone or combined with Mo at days 1, 5 and 7 for 1 or 2 days. Viable bacteria were plated for CFU. Video and scanning electro microscopy were carried out. The components of the biofilm matrix were analyzed biochemically, and purified.
Results: Both MAC 104 and A5 biofilms were not affected by adding of resting Mo or activated Mo. Mo in contact with the biofilm appear "hyper-activated" according to EM, O2- and NO production data. Mo on biofilms had impaired ability to ingest and kill M.smegmatis. The effect could be reproduced by exposed Mo to purified biofilm fractions. While contact with Mo upon seeding of biofilm led to decrease in bacterial load, established biofilms were not affected by macrophages. Presence of biofilm was associated with increased Mo apoptosis. Several proteins specific for M.avium biofilm and extracellular DNA were identified. A couple of the proteins were associated with apoptosis. Conclusion: M. avium biofilm is harmful to both, resting and activated Mo, inducing apoptosis. Mo exposed to biofilm are functionally impaired. Investigation of the effect of biofilm components on Mo may provide information regarding therapy.
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Role of Francisella tularensis regulatory factors and pathogenicity island genes in NADPH oxidase inhibition

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Francisella tularensis is a gram-negative, facultative intracellular pathogen and is the etiologic agent of the disease tularemia. *F. tularensis* is capable of infecting a variety of cell types including neutrophils. We demonstrated previously that evasion of intracellular killing is due, in part, to inhibition of NADPH oxidase assembly and activation followed by phagosome escape and bacterial persistence in the neutrophil cytosol. In addition, this pathogen differs from most other bacteria in its ability to impair neutrophil activation by heterologous stimuli. *F. tularensis* virulence genes required for evasion of oxidative host defense are not well defined. A small number of regulatory factors control virulence gene expression in this organism including MglA, SspA, MigR and FevR. Published data indicate that these factors positivity regulate expression of approximately 100 genes including but not limited to those in the Francisella pathogenicity island (FPI). Genes in the FPI are essential for phagosome escape, but whether they contribute to blockade of the respiratory burst is unknown. Using mutants generated in several *Francisella* strains

including the fully virulent type A strain Schu S4, we show here that all four regulatory factors are required for inhibition of NADPH oxidase activity, whereas *iglI* and *iglJ*, as well as other genes in the FPI tested to date are not. In addition, we show that blockade of the respiratory burst is dynamic and is modulated by bacterial growth conditions. Taken together, these findings suggest that genes required for NADPH oxidase inhibition may reside outside the FPI and as such may be distinct from those that mediate phagosome escape. This study was supported by NIAID R01AI073835 and a VA level 2 Career Development Award.

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Chlorpromazine-induced Relocalization of HIV-Gag+CD81+ Vesicles to the Macrophage Surface

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The presence of HIV reservoirs is a major barrier for achieving sterilizing immunity. Infectious HIV particles accumulate and are maintained within multivesicular bodies (MVBs) of cultured macrophages for extended periods of time. For transmission HIV particles must reach the cell surface yet, little is known about the mechanisms. Except in cases of direct macrophage-to-target cell transfer, HIV budding at the macrophage cell surface is rarely detected. The objective of this study was to test the hypothesis that dynamic rearrangement of the cytoskeleton and membrane fusion is required for HIV particles in MVBs to reach the cell surface. Macrophages infected with wildtype or GFP-tagged HIV were treated with chlorpromazine (CPZ), an agent that disrupts clathrin and other specific cytoskeletal proteins and analyzed by microscopic and biochemical methods.

Exposure of HIV-infected macrophages to CPZ led to the redistribution of clathrin and HIV Gag from intracellular sites to the cell periphery. Further analyses revealed that the tetraspanin CD81, a marker for MVBs, was closely associated with Gag+ vacuoles. Biochemical analyses confirmed that CD81 and HIV Gag form a protein complex. Interestingly, in HIV-infected, but not in uninfected macrophages, treatment with CPZ induced a dramatic relocalization of Gag+CD81+ MVBs from internal sites to the plasma membrane. Electron microscopic examination of infected macrophages treated with CPZ revealed an abundance of viral particles and intralumenal vesicles at the surface. Moreover, a significant difference in the distance of MVB localization from the plasma membrane surface in CPZ-treated and untreated HIV-infected macrophages was found. Lastly, treatment of HIV-infected macrophages with CPZ led to greater release of viral particles.

These findings support a model implicating the role of the cytoskeleton in HIV particle-laden MVBs dynamics. With a better understanding of the regulatory mechanisms strategies to enhance the presentation of HIV-infected macrophages to the immune system may be devised.

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Real Time Examination of the Effects of Bacillus anthracis Toxins on Spore-induced Neutrophil Recruitment in vivo

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The spore-forming bacterium *Bacillus anthracis* is the causative agent of anthrax. Its primary virulence factors include a tripartite toxin that includes two enzymatic effectors, lethal factor

and edema factor. Data on the toxins' function in pathogenesis has been contradictory; some studies suggest they activate phagocytes whereas others imply inhibition. In an attempt to rectify these apparently conflicting data we aimed to develop methods for studying phagocyte function *in vivo*. We hypothesize that *B. anthracis* toxins act to down-regulate the inflammatory response. Peripheral blood polymorphonuclear leukocytes (PMN), or neutrophils, were isolated from luciferase (luc) expressing mice and then injected into congenic luc -/- mice. These mice were then subcutaneously infected in the ear with *B. anthracis* 7702 (wt), a triple toxin knockout *B. anthracis* (TKO), a non-pathogenic *Escherichia coli*, 0.9% saline, or remained untreated. The influx of light-producing neutrophils into the site of injection over time was measured non-invasively using an IVIS Spectrum camera. An initial peak of luminescence followed by a secondary peak was observed for all experimental stimuli. The timing of the peaks was defendant on the type of bacteria used for infection; the time to initial peak and secondary peak for the wt and TKO was 10 and 20 hr and the timing for *E. coli* was 5 and 15 hr, respectively. The magnitude of luminescence was highest for the TKO strain, wt was notably lower, and the lowest bacteria-induced signal was from *E. coli*. The data described above suggest that *B. anthracis* toxins attenuate the chemotaxis of neutrophils to the site of cutaneous infection *in vivo*. Neutrophil recruitment occurs in a biphasic manner independently of the inflammatory stimuli. However, the timing and magnitude of the neutrophil influx is dependent on the stimuli. In particular, it took longer for neutrophils to chemotax towards *B. anthracis* spores, perhaps reflecting the time required for spores to germinate and begin vegetative growth.

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Characterization the protective mucus layer of the intestine

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The mammalian intestine harbors complex societies of beneficial bacteria coexisting with the host. The first line of defense preventing these micro-organisms to invade the underlying epithelia is a thick mucus layer with the heavily O-glycosylated MUC2 mucin as the main structural component. The mucus barrier is made up by two layers with similar composition. The outer layer is loose and easy removable while the inner is more dense and firmly adherent to the underlying epithelia. The inner layer is impermeable to bacteria and therefore separates the micro-organisms that resides in the lumen from the epithelia cells. In order to obtain a greater understanding of the regulation, function and structure of this barrier we analyzed the mucus using proteomics to find additional components. Interesting proteins where chosen, cloned and expressed in mammalian cell system in order to investigate the protein properties. AGR2 belongs to the protein disulfide isomerase family, and has been showed important for MUC2 production. AGR2 interact with MUC2 and is probably essential for correct folding of the MUC2 mucin. FCGBP is a large, highly repetitive protein that contains 13 vWD domains. 11 of these domains contain an autocatalytic cleavage site that after cleavage forms a new reactive C-terminus. FCGBP is found covalently bound to the MUC2 mucin and we believe that FCGBP acts as a cross linker of the mucus gel. ZG16 is a small lectin like protein able to bind peptidoglycan as well as gram positive bacteria. Even though ZG16 is not directly bactericidal we postulate that ZG16 plays an important role of the protective properties of the mucus gel.

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Phagosomal signaling by Borrelia burgdorferi in human monocytes involves TLR2-TLR8 cooperativity and TLR8 mediated transcription of IFN-β

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Phagocytosed *Borrelia burgdorferi* (Bb) induces inflammatory signals that differ both quantitatively and qualitatively from those generated by spirochetal lipoproteins interacting with TLR1/2 on the surface of human monocytes. In contrast to spirochetal lipoproteins, internalized spirochetes induce transcription of IFN-β. Using specific inhibitory immunoregulatory DNA sequences (IRS) for TLR-7 (IRS661), TLR-8 (IRS957), and TLR-9 (IRS869), we show that TLR8 is required for transcription of IFN-β and contributes to production of TNF-α, IL-6 and IL-10 in Bb-stimulated, highly purified human monocytes. Using confocal microscopy we provide visual evidence that in human monocytes, live Bb is initially sensed by TLR2 during binding and internalization, whereas fully degraded bacteria interact with both TLR2 and TLR8 in the phagosome at later stages. In parallel experiments we demonstrate that phagocytosed Bb also induces transcription of TLR2, TLR8 and IRF7, and that transcription of TLR8 and IRF7 is abrogated by IRS957. Lastly, we show by microscopy that IRF7 is translocated into the nucleus of Bb-infected monocytes, suggesting its activation through phosphorylation of constitutively expressed IRF7 residing in the cytosol. We conclude that in the case of human monocytes, phagosomal signaling in response to Bb involves a sequential and cooperative interaction between TLR2 and TLR8, while transcription of IFN-β occurs exclusively through TLR8 and involves IRF7. This study is the first to describe direct involvement of TLR8 in the activation of human cells after infection with an extracellular bacterium and confirms that TLR cross-talk occurs upon stimulation with a whole organism. This work was supported by Public Health Service grants AI-62439-03 (JCS), AI-38894 (JDR), AI-29735 (JDR) and M01RR006192 (UCHC).

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NMR reveals that phenylalanine126 in human sMD-2 affects endotoxin fatty acyl chain positioning in a TLR4-activating complex of endotoxin.MD-2

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Monomeric (hexaacylated) endotoxin (E)×MD-2 complexes are potent TLR4 agonists or antagonists depending on the structure of E and/or MD-2. Structural data derived from X-ray crystallography have been reported on the binary complexes of antagonists bound to MD-2 and ternary complex of agonist endotoxin bound to MD-2/TLR4 ectodomain. However, no structural data on a potent TLR4-activating E×MD-2 complex have been reported. Using a combination of metal chelation and size exclusion chromatographies, purified E:wild-type sMD-2 (TLR4 agonist) or E×F126A sMD-2 (TLR4 antagonist) complexes containing metabolically [^{13}C]-labeled endotoxin with ^{13}C-labeling at alternating carbon positions of the attached fatty acyl chains, have been generated in quantities suitable for NMR studies. High resolution ^{13}C/^{1}H HSQC spectra were acquired by focusing on the methyl region of the various terminal $^{13}CH_3$ groups of the fatty acyl chains in E×MD-2. Comparison of ^{13}C/^{1}H HSQC spectra

from the wild-type and mutant (F126A) E×MD-2 complexes suggests that: (1) re-orientation of F126 observed in the crystal structure of hexaacylated E×MD-2×TLR4 (ectodomain) ternary complex is induced in the E×MD-2 complex prior to engagement with TLR4 and (2) chemical shift perturbation of the $^{13}CH_3$ groups suggests that F126A mutation leads to repositioning of the acyl chains due to the removal of the interaction between the aromatic ring and endotoxin. Studies are in progress to compare the susceptibility of the terminal $^{13}CH_3$ groups in wild-type vs. F126A MD-2 complexes to quenching by a paramagnetic chelated gadolinium reagent to determine if the availability of a less sequestered fatty acyl chain for interaction with TLR4 is modified by the F126A mutation of MD-2.

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Replication of Type A and B Strains of Francisella tularensis in Interferon gamma Activated Human Macrophages

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Background

Francisella tularensis (Ft) is a small Gram-negative coccobacillus and the causative agent of tularemia. Only Ft subspecies *tularensis* (type A) and subspecies *holarctica* (type B) cause disease in humans, whereas both these Ft subspecies and the related bacterium, *F. novicida* cause a tularemia like disease in mice. Macrophages are a major reservoir of Ft *in vivo*. Studies of mice that survive infection with this organism indicate an important role for IFNγ in host survival. In the mouse model of infection and in primary murine macrophages *in vitro*, IFNγ mediated induction of iNOS plays a dominant role in bacterial killing, however the extent to which IFNγ activated human macrophages control this pathogen is not well defined.

Methods

Colony Forming Units (CFU) and microscopy analyses were used to compare the growth of Ft subspecies *tularensis* strain Schu S4, *holarctica* strain 1547 and LVS, and *F. novicida* strain U112 in IFNγ activated (100U/ml for 24hr) human monocyte-derived macrophages (MDM) and THP-1 cells. A Lucigenin enhanced chemiluminescent assay has been employed to characterize the ability of these strains to disrupt PMA stimulated Reactive Oxygen Species (ROS) production in both resting and IFNγ activated MDM.

Results

Our findings show that treatment of MDM or THP-1 cells with IFNγ prior to infection with *F. novicida* was bacteriostatic, but did not confer intracellular killing. In marked contrast, treatment of human macrophages with IFNγ had very little effect on LVS, 1547, or Schu S4. Moreover, stimulation of MDM with Salmonella LPS in addition to IFNγ did not enhance the effects of IFNγ treatment alone. Furthermore, we demonstrate that *F. novicida* is not capable of inhibiting PMA stimulated ROS production in IFNγ activated MDM.

Conclusions

These data are noteworthy since they define distinct features of virulent human pathogenic strains of Ft, and further suggest that Ft may impair IFNγ signaling and alter the activation phenotype of a macrophage as part of its virulence strategy. Specifically, type A and B strains are able to replicate and inhibit PMA stimulated ROS in IFNγ activated MDM, while *F. novicida* is not. This clear difference in the sensitivity of *F. novicida* to IFNγ activation further supports the notion that despite its historical use as a model, due to its ease of genetic manipulation and non BSL-3 level work, not all findings with this organism may translate to type A and B *Francisella*. Experiments designed to investigate the basis for this sensitivity are underway.

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Stage-specific pathways of Leishmania entry into macrophages

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Leishmania spp. have a life cycle with two stages: the promastigote, inoculated in the skin by the sand fly vector and the amastigote, the replicative form of the parasite found in macrophages. Each parasite stage must undergo phagocytosis by macrophages during the parasite life cycle. We previously showed that caveolae, a subset of cholesterol-rich membrane lipid rafts, facilitate both uptake of virulent promastigotes and subsequent intracellular survival at least in part by delaying parasitophorous vacuole (PV)-lysosome fusion. Amastigotes differ from promastigotes in both their surface composition and their resistance to the harsh phagolysosome environment. We hypothesized that the parasite stages also differ in their route of macrophage entry and in the rate of PV maturation. Accordingly, transient disruption of macrophage lipid rafts decreased the entry of promastigotes, but not amastigotes, into macrophages ($P<0.001$). Promastigote-containing PVs transiently associated with EEA-1and Rab5 but dissociated after 15 min infection, whereas EEA-1 and Rab5 remained in amastigote-containing PVs for at least 2h. Coinciding with their conversion into amastigotes, the percentage of LAMP-1+ promastigotes increased from 20% at 1h to 46% by 24h ($P = < 0.001$, Chi square). In contrast, most amastigotes were LAMP-1+ at both 1 and 24h. Furthermore, lipid raft disruption increased LAMP-1 recruitment to promastigote, but not to amastigote-containing compartments. Our results support a model in which entry through caveolae channels promastigotes through a path that delays PV-lyosome fusion. Amastigotes, the more resilient parasite form, enter through a pathway leading to abnormal phagosome maturation that rapidly acquired lysosomal markers. This work was supported by a Career Developmental Award (CDA-2) from the Departments of Veterans Affairs (NER) and Merit Review grants (L-AHA) and (MEW) from the Department of Veterans' Affairs (MEW).

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C-type lectin receptors in pulmonary Francisella infection

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Recent studies from our laboratory have shown that in the absence of any exo- or endotoxin activity of the pathogen, lethal pulmonary infection of mice with the intracellular, Gram negative bacterium and a CDC category A bioterrorism agent *Francisella tularensis* is associated with a deregulated host response culminating in severe sepsis. As innate immune responses play a major role in development of sepsis, we wanted to examine a previously undetermined role of an innate immune receptor family, the C-type lectin receptors (CLRs), in Francisella infection induced sepsis. Real-time RT-PCR and immunofluorescence (IF) microscopy analyses showed differential upregulation of several CLRs at both transcript and protein levels, in the lungs of infected mice. Interestingly, Galectin-3 (Gal-3), a host CLR, normally found intracellularly, was observed to be released extracellularly in lungs of infected mice during the septic phase of lethal pulmonary infection with wild-type (WT) Francisella but not in the mice vaccinated with an attenuated mutant and protected from septic challenge with the WT organisms. Furthermore, mice deficient in Gal-3 (Gal-$3^{-/-}$ mice) showed reduced levels of inflammatory cytokines in infected lungs as compared to their WT counterparts. In-vitro studies showed that pre-treatment of bone-marrow derived macrophages with recombinant galectin-3 predisposes these cells to produce enhanced levels of inflammatory cytokines in response to Francisella infection. Taken together, our studies show that Gal-3, an intracellular CLR lacking a secretion signal

sequence, is released extracellularly in lungs of mice during septic phase of respiratory Francisella infection and plays an immunostimulatory role, possibly contributing to the development of sepsis during this infection.

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Fatty acid binding protein facilitation of Mycobacterium tuberculosis growth and survival in macrophages

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The fatty acid-binding proteins (FABPs) A-FABP and E-FABP are expressed in macrophages where they play a role in both the regulation of cholesterol trafficking and inflammatory function. We demonstrated previously that these FABPs act as negative regulators of PPARγ function. Thus, the absence of FABP expression, macrophages display enhanced PPARγ activity, resulting in enhanced expression of cholesterol trafficking proteins and reduced expression of inflammatory cytokines. Recent studies have demonstrated host cholesterol dependency of *Mycobacterium tuberculosis (M. tuberculosis)* for its attachment and intracellular survival in macrophages. In this study we identified a role of FABPs in uptake, early survival and intracellular growth of *M. tuberculosis.* Uptake, early survival and subsequent intracellular growth were greatly reduced in both A-FABP and E-FABP-deficient macrophages as compared to wild-type macrophages. To verify the role of FABPs, wild-type macrophages were pre-treated with a synthetic FABP inhibitor, HTS01037, which resulted in similar defects in uptake, early survival and subsequent intracellular growth as was observed in the FABP-deficient cells. Binding assays, performed in the presence of cytochalasin D, a phagocytic inhibitor, showed no defect in attachment of bacteria by FABP-deficient macrophages. Previous studies have found that IFN-γ treatment of macrophages limits the available intracellular nutrient source for *M. tuberculosis* and increases the cholesterol dependency. We found that IFN-γ treatment of wild-type macrophages reduced uptake and early survival but did not impact subsequent intracellular growth, whereas, IFN-γ treatment of E-FABP-deficient macrophages decreased both uptake and intracellular growth. Overall, our results demonstrate that FABPs support *M. tuberculosis* growth and survival, possibly via enhancement of macrophage cellular cholesterol content. Interestingly, *M. tuberculosis* infection of wild-type macrophages was found to enhance FABP expression, which provides the pathogen with a mechanism by which it can improve its potential for survival.

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A Role for PTEN and IRAK-M in PGE2-Induced Immunosuppression Post-Bone Marrow Transplant

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Hematopoietic stem cell transplant patients are often susceptible to lung infections for prolonged periods, despite complete immune reconstitution. In a mouse model of syngeneic bone marrow transplant (BMT), we previously reported that prostaglandin E2 (PGE_2) is overproduced in lungs of BMT mice, impairing host defense against *Pseudomonas aeruginosa*. This impairment in host defense is also marked by diminished alveolar macrophage (AM) phagocytosis, bacterial killing, and production of TNF-α. However, a mechanism by which PGE_2 overproduction suppresses lung innate immunity post-BMT is unknown. Phosphatase and tensin homolog on chromosome ten (PTEN) and interleukin-1 receptor associated kinase (IRAK)

-M are known inhibitors of AM phagocytosis/killing and MyD88-dependent-TLR/IL-1R signaling respectively. Therefore, we sought to determine whether these two molecules were involved in PGE_2-induced immunosuppression post-BMT. We found that AMs from BMT (WT>WT) mice displayed elevated PTEN activity, and this was related to increased PGE_2 signaling. Furthermore, BMT AMs displayed elevated IRAK-M expression, which was significantly reduced by inhibiting endogenous PGE_2 production in AMs. Inhibiting PTEN activity restored phagocytic ability in BMT AMs. Likewise, transplanting WT mice with IRAK-M -/- bone marrow improved host defense against P. aeruginosa and AM phagocytosis, killing and production of TNF-α. Preliminary evidence suggests that PTEN activity is necessary for PGE_2-mediated IRAK-M elevation. Overall, these data suggest that overproduction of PGE_2 post-BMT may impair AM function by upregulating PTEN activity and IRAK-M expression. Strategies to target these molecules may prove efficacious in limiting infections post-BMT.

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Effects of Interleukin-10 on gene expression in human monocytes and on genes induced by Neisseria meningitidis

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Introduction: *Neisseria meningitidis* (*Nm*) may cause fulminant septicaemia and a "cytokine storm". Interleukin-10 (IL-10) is the principal anti-inflammatory cytokine in plasma (J Exp Med 1996;184:51). *In vitro* exposure of human monocytes to Nm leads to expression of a huge set of differentially expressed genes (Infect Immun 2008;76:2685). **Aim:** To explore the effects of IL-10 on gene expression induced by *Nm* in human monocytes *in vitro*. **Materials and methods:** Elutriation-purified, cryopreserved human monocytes (J Immunol Methods 2001;255:45) from 5 donors were seeded in microtiter plates ($0{,}75 * 10^6$ cells/ml per well) and exposed to $1{,}0 * 10^6$*Nm*/ml or vehicle in the presence and absence of 25ng/mL rIL-10 for 3 hours (37 C°, 5% CO_2). Cells and supernatants were harvested, total-RNA isolated (Qiazol method) and examined using Affymetrix Human Gene 1.0 ST Array. Statistics: Array Assist (Iobion Informatics), Partek Genomics Suite (Partek Incorporated), each donor its own control. Filtering: fold change≥ |2| and p -value < 0.05.

Results: *Nm* induced 1170 differentially expressed genes. 34 genes up-regulated (FC range 2 to 24) ≥ |2|) ≤ |24|) by *Nm* had a reduced expression (FC range -1.4 to 6.2) in the presence of IL-10. 18 of the 34 genes are directly related to inflammatory responses. Genes involved in systemic inflammatory pathophysiology like IFN- γ, tissue factor, TNF-α, CXCL11 and IL-12 were among the affected. Presence of IL-10 *with* Nm reduced the concentration of proteins in culture supernatants (measured with Luminex technology) with 72% to 40%. 43 genes down-regulated by Nm had an increased expression (FC≥ |2|) in the presence of IL-10. Among these were CD14, thrombospondin and Siglec9. IL-10 alone induced 173 differentially expressed genes. Principal component analysis and heatmaps will further underscore the results. **Conclusions:** Microarray analysis confirms IL-10 to be a potent anti-inflammatory stimulus to human monocytes in response to *Neisseria meningitidis*.

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Differential role of the endogenous microbiota in the promotion of colitis and intestinal colorectal cancer

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Although the etiology of colitis-associated colon cancer (CAC) remains elusive, innate host sensors have been shown to modulate the pathogenesis. Here, we determine the impact of the microbiota on the development of CAC. WT and *Il10*$^{-/-}$ germ-free mice (n=19) were conventionalized under specific pathogen-free (SPF) conditions or mono-associated with *Escherichia coli* NC101 (*E. coli) or Enterococcus faecalis* (*E. faecalis*). Tumors were induced by intraperitoneal injection of azoxymethane (AOM) once a week for six weeks and then followed for 14 weeks. Colonoscopy was performed to follow the development of inflammation and colorectal tumors. Inflammatory mediators were profiled using an inflammatory PCR array (SABiosciences). DNA was isolated from the stools and subjected to terminal restriction fragment length polymorphism (TRFLP) analysis with terminal restriction fragments (TRF) peak height analyzed using PRIMER VI and Stata. TRFLP analysis demonstrates a reduction of microbial diversity in *Il10*$^{-/-}$ mice compared to WT mice, with specific TRFs associated with development of colorectal cancer. Interestingly, although *E. faecalis*-monoassociated *Il10*$^{-/-}$ mice developed colitis comparable to conventionalized or *E. coli* - monoassociated *Il10*$^{-/-}$ mice (ave. score = 3.2/4), tumor numbers were reduced by more than 40 fold in *E. faecalis*- monoassociated *Il10*$^{-/-}$ mice. Gene array analysis revealed a similar gene profile between *E. coli*- and *E. faecalis*-monoassociated *Il10*$^{-/-}$ mice, including the key inflammatory mediators IL-6, IFNg, Il-12p40, IL-23p19, IL-17 and TNF. Interestingly, PCR analysis showed the presence of the genomic island *pks* encoding for the polyketide-peptide genotoxin Colibactin in *E. coli* NC101 but not in *E. faecalis* . These findings indicate that bacterial composition and specific bacterial genes strongly influences the development of CAC in *Il10*$^{-/-}$ mice and suggest that chronic inflammation is not sufficient to promote CAC development. Altering the composition of the microbiota may represent a novel means by which to modulate development of CAC in IBD patients. NIH DK73338 and DK47700

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IL-17 enhances mycobacterium induction of IL-6 expression in human primary blood macrophages

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Tuberculosis is still a major threat to the world. Although many people received *Mycobacterium bovis* bacilli Calmette-Guerin (BCG) vaccination, there are still 12 million new cases and 2 million people died from *Mycobacterial tuberculosis*(MTB) infection worldwide every year. Recently, the incidence of multidrug-resistant MTB infections is increasing globally and the treatment for that is complicated. Additional understanding of the pathogenesis and immune responses may contribute to the development of more effective vaccines and new therapeutics for MTB.

IL-17 is a cytokine produced by a specific subgroup of Tlymphocytes known as T-Helper 17 (TH17) cell. Apart from its important role in developing adaptive immunity, recent studies showed that TH-17 cells and IL-17 also participated in innate immunity against different pathogens including mycobacteria. Since the detailed mechanisms are still unknown, we investigated whether IL-17 regulates the expression of cytokines that are critical in immune defense against mycobcateria.

Primary human blood macrophages were treated with or without IL-17A for 24 hours followed by the BCG stimulation. The results demonstrated the production of IL-6 was elevated in the primary blood macrophages treated with both IL-17 and BCG compared to those cells treated with BCG or IL-17A alone. We further illustrated that IL-17 could stabilize IL-6 mRNA, with consequent production of more secreted IL-6 protein. Additionally, IL-17 was found to interact with BCG-induced TNF-alpha to upregulate IL-6 production. Since IL-6 is essential for the generation of TH17 cells, the induction of IL-6 by IL-17 may contribute to the positive regulatory loop for the generation of

TH17 cells. We are currently investigating the detailed pathways involved. By these mechanisms, IL-17 could enhance the pathogen-induced proinflammatory cytokine responses and play a role in coordinating immune response against mycobacterial infection.

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HIV-1 trans-activator protein dysregulates IFN-gamma signaling: a role for STAT-1 in autophagy

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HIV-1 transactivator protein, Tat, was first identified as an activator of HIV-1 replication through its upregulation and binding of transcription factors complexes. Recent reports including ours showed that HIV-1 Tat was also found to play an important role in the dysregulation of cytokine expression and cell signaling (AIDS 2010) as well as induction of apoptosis in T-cells. Autophagy is a specialized type of cell death process responsible for self-digestion and degradation mechanism to recycle the cytosolic contents including macromolecules and cellular organelles resulting in self-repair and conservation for survival. Recent studies demonstrated that autophagosomes induced by IFN-gamma also participate in immune defense by processing the nuclear antigen for presentation and killing of intracellular pathogens. As we previously demonstrated that HIV-1 Tat perturbs IFN-gamma signaling via suppression of STAT1 phosphorylation and consequently inhibits MHC-II antigen expression, we postulate Tat may play a role in regulating autophagy as part of cellular defense against pathogens.

We induced autophagy in human primary blood macrophages by treating the cells with IFN-gamma for 8 to 16 hours. To demonstrate the role of STAT1 in IFN-gamma induced autophagy, siRNA specific to STAT1 and a chemical inhibitor of STAT1 were added to the cells prior to IFN-gamma stimulation. This was followed by assaying the protein expression of LC3. The results showed that knock down of STAT1 expression could suppress IFN-gamma -induced LC3 expression and autophagosome formation, thus confirming the role of IFN-gamma and STAT1 in autophagy. We further examined the effects of HIV-1 Tat on IFN-gamma -induced autophagy. Here we demonstrated that recombinant HIV-1 Tat suppressed IFN-gamma –induced upregulation of LC3 expression and formation of autophagosomes. Furthermore, HIV-1 Tat restricted the capturing of mycobacterium by autophagosomes.

In conclusion, HIV-1 Tat suppressed the induction of autophagy-associated genes and inhibited the formation of autophagosomes. Perturbation of such cellular processes by HIV-1 would impair effective containment of the invading pathogens, hence providing a favorable environment for the opportunistic microbes in HIV-infected individuals.

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Innate Immunostimulatory Lipopeptides of Staphylococcus aureus as TLR2 Ligands; Prediction with mRNA Expression, Chemical Synthesis, and TLR2-mediated NK Activation

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Bacterial lipoproteins/lipopeptides are generally accepted to be Toll-like receptors 2 (TLR2)

ligands, although the diversity of the compounds from microorganisms were reported [1]. The structures of TLR2 ligands from Gram-positive bacteria, however, had not been clearly shown because of the difficulties in isolation of the pure ligands from natural sources, until recent results including ours. In this study, we targeted lipoproteins derived from *Staphylococcus aureus* strain NCTC 8325. We selected 58 candidate lipoproteins, which were predicted from genome sequence with DOLOP (Database of bacterial Lipoproteins), and searched their lipoproteins based on the result of expressed level of the mRNA. The lipoproteins were predicted to have diacylated *S*-(2,3-dihydroxypropyl)cysteine from their biosynthetic pathway. We synthesized sixteen lipopeptide fragments with the combination of solid-phase and solution-phase synthesis, and observed TLR2 recognition and immunostimulatory activities. The lipopeptides showed TNF-α inducing activity in peripheral blood mononuclear cells (PBMC). They also induced NF-κB activation in Ba/mTLR2 cells, and these results indicated that the activation by the synthetic fragments is mediated by TLR2. It was also shown that activation potencies varied depending on the amino acid sequence, and the lipopeptides with hydrophobic peptide moieties showed relatively weaker activities [2] . The peptide sequence of TLR2-agonistic lipopeptides also critically affected the potential for NK activation [3].

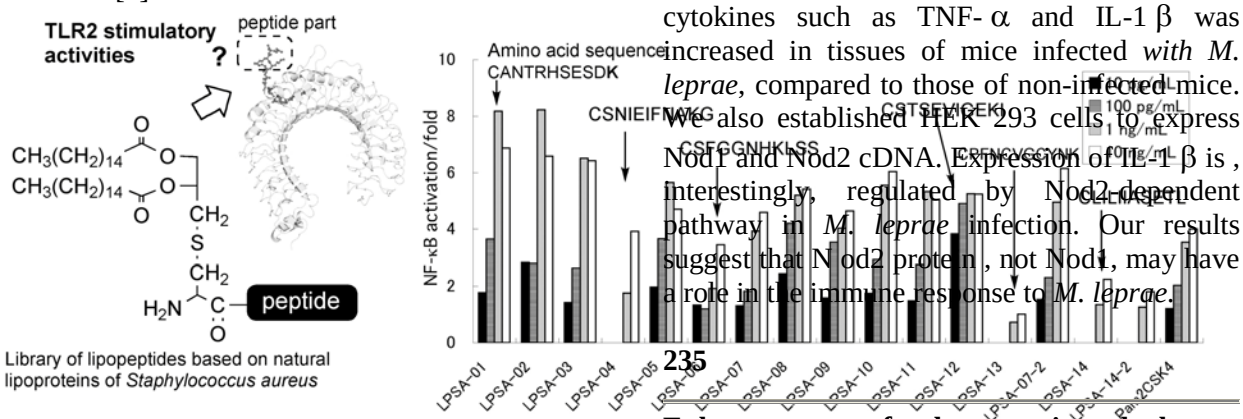

Figure. TLR2 stimulatory activities of synthesized lipopeptides. NF-κB dependent luciferase activities in murine TLR2 expressing Ba/F3 cells (Ba/mTLR2) was determined.

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Nod2, not Nod1 Regulates the Expression of IL-1beta in the Immune Response of Host against M. leprae.

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The nucleotide-oligomerization domain (NOD) is an important molecule involved in host defense against bacterial infection . In order to determine the function of Nod1 and Nod2 in response of host against *M. leprae* infection, we measured the Nod1 and Nod2 mRNA level in tissues of nu/nu mouse and RAW 264.7 cells infected with *M. leprae*. The expression of NOD1 and NOD2 mRNA was induced in tissues of nu/nu mice infected with *M. leprae*. In addition, the expression of inflammatory cytokines such as TNF-α and IL-1β was increased in tissues of mice infected *with M. leprae*, compared to those of non-infected mice. We also established HEK 293 cells to express Nod1 and Nod2 cDNA. Expression of IL-1β is , interestingly, regulated by Nod2-dependent pathway in *M. leprae* infection. Our results suggest that Nod2 protein , not Nod1, may have a role in the immune response to *M. leprae*.

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Enhancement of the porcine leukocyte oxidative burst by feeding the B1 peptide to weaned pigs

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A gram-positive bacterium, *B. texasporus* (ATCC PTA-5854), has been isolated and found to produce BT, a group of related cation amphipathic peptides of 13 amino acid residues. The BT peptide has been previously shown to enhance the leukocyte response in chickens when included in a feed ration. In the present study, weaned pigs were fed a normal feed ration with the addition of 24 ppm of the BT peptide for 7 days. Control pigs were fed the same ration without the inclusion of the peptide. Pigs were bled on Days 0, 3, 5, and 7 of the study and leukocytes were isolated using established methods. Production of an oxidative burst along with cellular degranulation were assayed. On all days measured, cellular degranulation was not found to be different between leukocytes from control and peptide-fed pigs. The production of an oxidative burst in leukocytes from peptide- fed pigs was found to be significantly ($P < 0.05$) higher than produced by leukocytes from control pigs on Days 3, 5, and 7. The data presented here, in conjunction with data showing reductions in *Salmonella* colonization in pigs fed the peptide, indicate that the BT peptide may represent an alternative immunomodulatory agent to control food-borne pathogens in swine.

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The Effects of Ethanol on Macrophage Fcgamma-Receptor Mediated Phagocytosis

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It is estimated that 3.8% of all global deaths and 4.6% of global disease, including the reduction of healthy living years, is attributed to alcohol (ethanol) consumption. Our initial studies revealed that acute *in vivo* or *in vitro* ethanol exposure decreases alveolar macrophage phagocytosis of *Pseudomonas aeruginosa.* To investigate a single receptor pathway, we chose to focus our studies on Fcγ-receptor (FcγR) mediated phagocytosis. FcγR-mediated phagocytosis of IgG coated beads had decreased F-actin recruitment to the site of the phagosome by 40% in the ethanol treated groups of both murine alveolar macrophages and the RAW264.7 murine macrophage cell line. Prior to actin polymerization, focal adhesion molecules paxillin and vinculin are recruited to the phagosome. Ten minutes of FcγR-mediated phagocytosis induces a 2-fold increase in the phosphorylation of vinculin in the control group, which is not observed in the ethanol treated cells. In contrast, a similar 2-fold increase in paxillin phosphorylation following FcγR activation occurs independent of ethanol exposure. Small GTPases are known to regulate normal actin polymerization. RAW264.7 cells exposed to NSC23766 (a Rac1 inhibitor) show 45% suppression in actin polymerization to the site of the phagosome, regardless of ethanol exposure. This underscores the importance of normal Rac activity during FcγR-mediated phagocytosis. Rac is active in its GTP bound form, and our study reveals that cells exposed to ethanol exhibit a decrease of 50% in Rac activity prior to phagocytic stimulus. Following 15 minutes of IgG-coated bead phagocytosis Rac activation increases by 50% compared to 175% in control conditions. Attenuated FcγR-mediated phagocytosis in subjects who consume alcohol would render them more vulnerable to infection, as well as potentially prolonging infection. (Supported by NIH R01 AA012034 (EJK), NIH F31AA017027 (JK), NIH F31AA017032 (ELM), Illinois Excellence in Academic Medicine Grant and Ralph and Marian C. Falk Research Trust)

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Theromodynamic analysis of binding between LL-37 and LPS, LTA. ODN and HA
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OBJECTIVES: The antimicrobial peptide cathalicidin LL-37, a host defense peptide derived from leukocytes and epithelial cells, plays a crucial role in innate and adaptive immunity. LL-37 is a multifunctional peptide with broad spectrum of antimicrobial activities, with the ability to prevent the immunostimulatory effects of bacterial wall molecules such as lipopolysacharide (LPS) and lipoteichoic acid (LTA). Additional reported activities of LL-37 include chemoattractant function, inhibition of neutrophil apoptosis, and stimulation of angiogenesis, tissue regeneration and cytokine release. Cellular production of LL-37 is affected by multiple factors, including bacterial product, host cytokines, availability of oxygen, and sun exposure through the activation of CAP-18 gene expression by vitamin D_3.
RESULTS: The aim of this study was to determine the binding of LL-37 to LPS, LTA, HA (hyaluronic acid) and ODN (oligodeoxynuclotides). The thermodynamic of the binding interaction was investigated by isothermal titration calorimetry (ITC). The ITC results shows that the interaction between LL-37 and investigated stimulators of Toll-like receptors are exothermic. The interaction between LL-37 and HA or ODN is via direct binding and LL-37 binds to HA and ODN at one set of sites. The calorimetric titration curve for binding LL-37 to LTA and LPS indicated at least two types of binding sites of differing affinities for each compound.
CONCLUSIONS: We reveal the nature of interactions between the LL-37, LPS, LTA, HA and ODN and show that LL-37 also modulated the inflammatory response to LPS and other agonist of TLR.

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Differential and Temporal Expression Patterns of Interferons and Th1/Th2 Cytokines in CpG Oligodeoxynucleotide and Double-stranded RNA Stimulated Chicken Monocytes
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Toll-like receptors (TLRs) recognize microbial components and initiate the innate immune responses that control microbial infections. TLR3 and TLR21 (the chicken equivalent to mammalian TLR9) recognize pathogen-derived nucleic acids, with TLR3 specific for viral double-stranded RNA and TLR21 for microbial CpG-DNA. Poly I:C and CpG-oligodeoxynucleotide (ODN), the ligands of TLR3 and TLR21, respectively, are known to differentially stimulate type-II interferon (IFN)-β and Th1 cytokine IFN-γ expression; and a combination of the two has shown to synergistically induce proinflammatory cytokines IL-1β and IL-6 and chemokines IL-8 and macrophage inflammatory protein (MIP)-1β in chicken monocytes. However, the interaction between poly I:C and CpG-ODN on the expressions of IFNs and Th1/Th2 cytokines has not been reported. Therefore, the present study was to investigate the effect of poly I:C, CpG-ODN, or a combination of the two on the expressions of IFN-α and IFN-β, Th1 cytokines IFN-γ and IL-12, and Th2 cytokines IL-4 and IL-10 in chicken monocytes. CpG-ODN was found to significantly up-regulate the expression of INF-γ, IL-10, and IL-12p35, but not IFN-α and IFN-β; whereas poly I:C induced, to a lesser extent, the expression of INF-γ, IFN-α and IFN-β, but not IL-12p35. Stimulation with the combination of CpG-ODN and poly I:C synergistically up-regulated IFN-γ and IL-10 expression. Similarly, IFN-α and IFN-β expression were also enhanced by the combinatory treatment of the two ligands. Finally, increased expression of IL-4 was observed in all groups at 24 h post treatment. The present data together with the results from our previous study provide strong evidence of the interaction between the TLR3 and TLR21 pathways; and this interaction promotes a Th1-based protective immune response in chicken monocytes. These results demonstrate the potential use of poly I:C and CpG-ODN as

innate immune modulators to reduce foodborne pathogens in poultry.

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mTOR signaling mediates Campylobacter jejuni induced colitis in IL 10-/- mice

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Campylobacter jejuni , a class B food borne pathogen, is a leading cause of worldwide enteritis. Unfortunately, the molecular mechanism responsible for *C. jejuni*-mediated pathogenesis is virtually unknown due to the lack of a robust in vivo infectious model. We previously demonstrated that germ-free IL-10-/- mice developed rapid (5 days) and severe intestinal inflammation following C. jejuni infection. In this study, we define the role of PI3K/mTOR signaling in *C. jejuni* induced pathogenesis. Germ-free IL-$10^{-/-}$; NF- k B^{EGFP} mice were infected with C. jejuni 81-176 (10^9 CFU/mouse) for 0-12 days. The PI3K (wortmannin 1.4mg/kg) and mTOR (rapamycin 1.5mg/kg) inhibitors were injected (i.p.) daily. *C. jejuni*-induced histological damage was reduced by ~90% in rapamycin-treated mice, which associated with inhibition of NF-kB-derived EGFP expression and p70s6 phosphorylation (T389) as measured by immunofluorescent and Western blot analysis. In addition, *C. jejuni* induced CXCL2, IL-17A and IL-1beta mRNA accumulation in IL-$10^{-/-}$; NF-κB^{EGFP} mice was attenuated by 75, 88, and 79%, respectively in rapamycin-treated, C. jejuni-infected IL-$10^{-/-}$; NF-κB^{EGFP} mice. Similarly, wortmannin treatment prevented C.jejuni-induced colitis and proinflammatory gene expression in IL-$10^{-/-}$; NF-κB^{EGFP} mice. Fluorescence in situ hybridization assay and 3-D imaging showed that rapamycin alleviated C. jejuni invasion into the colonic crypts of IL-$10^{-/-}$; NF- k B^{EGFP} mice. Intestinal and extra-intestinal (mesentery lymph nodes and spleen) C. jejuni count were strongly reduced in rapamycin-treated mice compared to untreated mice. In addition, *C. jejuni*-induced MPO+ cells (neutrophil) infiltration in the crypt were strongly reduced in rapamycin-treated IL-$10^{-/-}$ mice. Our findings demonstrate a novel role of PI3K/mTOR signaling in mediating *C. jejuni* induced colitis and suggest that targeting this signaling pathway may represent a potential mean to modulate pathogenesis.

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A Francisella Lipid A Mutant is Cleared by γ-Interferon and Confers Protective Immunity to Tularemia in Mice

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Francisella tularensis subspecies *tularensis* (Ft) is an intracellular Gram-negative bacterium and the causative agent of the severe human disease tularemia. *Francisella* lipid A, normally the biologically active component of

lipopolysaccharide (LPS) has no to low endotoxic activity.

A *Francisella tularensis* subspecies *novicida* (Fn) lipid A biosynthesis mutant was generated that lacked the 4´-phosphatase enzyme (LpxF). Analysis of lipid A isolated from this mutant strain showed the retention the phosphate moiety at the 4´ position and the N-linked fatty acid at the 3´ position on the diglucosamine backbone. This mutant was previously shown to be avirulent in the footpad infection model. Studies were undertaken to determine if this mutant was avirulent by more natural routes (aerosol and subcutaneous) of infection and to determine if this mutant could provide protection from a lethal WT challenge. All mice (C57BL/6) infected with the *lpxF*-null Fn mutant either by subcutaneous or the pulmonary route survived initial infection and subsequently developed protective immunity against a lethal wild type challenge.

To determine the mechanism of control of a primary infection by the *lpxF*-null mutant, C57BL/6 and RAG-$1^{-/-}$ mice were subcutaneously inoculated with the *lpxF*-null Fn mutant. All mice survived the primary infection indicating that T and B cells are dispensable for the control of *lpxF*-null Fn mutant infection. Components of the innate immune system, including TLR2, TLR4, caspase-1, MyD88, interferon-alpha (IFN-α) and interferon-gamma (IFN-γ) were also examined using knockout mice. Interestingly, only the IFN-γ knockout mice succumbed to a primary *lpxF*-null Fn mutant infection, highlighting the importance of IFN-γ production in control of Fn lipid A mutant infection. Work is currently underway to elucidate the source(s) of IFN-γ.

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Tobacco-induced alterations to Porphyromonas gingivalis fimbriae promote host colonization

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Introduction: Tobacco smokers are more susceptible to multiple bacterial infections, including the periodontal pathogen *Porphyromonas gingivalis*, and simultaneously exhibit a reduced mucosal inflammatory response to infection. We have recently shown that exposure to cigarette smoke extract (CSE) upregulates multiple *P. gingivalis* virulence factors, such as the major and minor fimbrial antigens (FimA and Mfa1 respectively) and capsular polysaccharides that are key in biofilm formation.

Hypothesis: CSE-exposure induces *P. gingivalis* FimA and Mfa1expression promoting biofilm formation through fimbrial interaction with specific *S. gordonii* adhesins (SspB and GAPDH) while simultaneously suppressing host inflammatory response.

Methods: CSE-induced phenotypic alterations were determined by electron microscopic and western blot analysis. *P. gingivalis-S. gordonii* biofilms were visualized by confocal laser scanning microscopy and analyzed using COMSTAT. The importance of FimA and Mfa1 in biofilm formation was confirmed using *P. gingivalis* fimbrial mutants and rSspB and rGAPDH dot blots. Cytokine induction on engagement of primary innate cells by FimA, Mfa1 and fimbrial mutants was determined by ELISA.

Results: CSE-exposure resulted in increased expression and surface availability of FimA, but not Mfa1, concomitant with increased binding to *S. gordonii* adhesins and increased dual-species biofilm formation that was, nevertheless, Mfa1-dependent. FimA was found to be a potent mediator of inflammatory suppression, inducing innate cell hyporesponsiveness (IL-6; TNF-α) to TLR2 and TLR4 agonists in an IRAK1-I□B□-dependent manner.

Conclusions: CSE-exposure increases fimbrial antigen expression in *P. gingivalis* and, subsequently, increases bacterial adhesion and

biofilm formation while concomitantly suppressing the pro-inflammatory response to this periodontal pathogen. These data help explain mechanistically increased susceptibility to *P. gingivalis* infection in tobacco smokers.

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Evasion of innate immunity by Granulibacter bethesdensis, an emerging Gram-negative bacterial pathogen

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A few members of the *Acetobacteraceae* have recently emerged as pathogens in subjects with inborn or iatrogenic immunodeficiencies. One of these pathogens, *Granulibacter bethesdensis*, has thus far caused disease only in individuals with chronic granulomatous disease (CGD), an immunodeficiency arising from defective production of reactive oxygen species (ROS) by phagocytes. To better understand this host tropism, we investigated the interaction of *G. bethesdensis* with polymorphonuclear leukocytes (PMN) from healthy subjects or patients with CGD. *G. bethesdensis* was internalized by normal and CGD PMN and the process required heat-labile serum components. *G. bethesdensis* was 10-100 times less potent than *E. coli* at stimulating ROS production by normal PMN. Despite this, there was moderate killing of the pathogen by these leukocytes (*G. bethesdensis* colony-forming units were reduced by ~50% over a 24-h co-culture with PMN). In contrast, CGD PMN were unable to kill the organism. *G. bethesdensis* was sensitive to H_2O_2 but resistant to cathelicidin, an antimicrobial peptide of PMN. Despite poor activation of the respiratory burst, microarray analysis of human PMN cultured with *G. bethesdensis* over a 24-h time period revealed complex cellular responses, including changes in transcripts encoding pro-inflammatory and anti-apoptotic molecules. *G. bethesdensis* inhibited constitutive and Fas-induced PMN apoptosis, while causing significant cytokine secretion. We propose that the resistance of *G. bethesdensis* to killing by CGD PMN and ability of the pathogen to prolong the PMN lifespan contribute to persistence *in vivo*.

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Mucosal Stromal Factors Regulate the Gastric Dendritic Cell Response to H. pylori

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Helicobacter pylori is the most common cause of chronic gastritis, peptic ulcer and gastric cancer. To elucidate the immunobiology of H. pylori gastritis, we have shown that gastric dendritic cells (DCs) are important initiators of the mucosal immune response to H pylori. Here we investigated whether the gastric microenvironment regulates DC function in human H. pylori infection. Using our established model of media derived from cell-free mucosal stroma (S-CM) to recapitulate the

mucosal environment, we detected higher levels of pro-inflammatory mediators in S-CM from healthy gastric mucosa compared to intestinal S-CM. However, DCs freshly isolated from gastric and intestinal mucosa were phenotypically and functionally identical. Primary gastric and intestinal DCs pulsed with H. pylori increased their expression of CD83 and induced T-cell IFN-γ secretion, but not proliferation, whereas H. pylori-pulsed, monocyte-derived DCs (MoDCs) induced strong T-cell proliferation and IFN-γ secretion. To further dissect the influence of the mucosal environment on the DC response to H. pylori, we generated MoDCs in the presence of either gastric or intestinal S-CM. S-CM-treated MoDCs expressed significantly lower levels of CD86 and HLA-DR compared to control DCs and lost their ability to increase CD80 and CD83 expression upon H. pylori stimulation. Antibody neutralization of TGF- β, a tolerogenic factor present in S-CM that contributes to intestinal macrophage anergy, did not reverse the inhibitory effects of gastric S-CM on DCs. The ability of H. pylori-pulsed DCs to initiate T-cell proliferation was significantly impaired for DCs generated in the presence of S-CM, and T-cell IFN-γ was also reduced, likely due to the concomitant decrease in DC IL-12p70 secretion, but a Th1 phenotype was still maintained. In conclusion, the mucosal DC response to H. pylori is down-regulated by soluble stromal factors. In gastric mucosa, this may contribute to the host's inability to clear H. pylori infection.

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MLCK deficiency protects from ethanol and burn injury induced intestinal damage and barrier dysfunction

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Ethanol exposure combined with burn injury increases morbidity and mortality and causes an aberrant immune response characterized by elevated levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6), and alterations of leukocyte infiltration to the wound and remote organs like the intestine. In the intestine, this response promotes morphological damage and changes in epithelial tight junctions promoting intestinal permeability. Using a mouse model of ethanol and burn injury, we hypothesized that deficiency in long myosin light chain kinase (MLCK), an enzyme important for barrier maintenance, would restore intestinal morphology and barrier function after injury. Six hours after ethanol exposure and burn injury, there were no differences between MLCK knockout and wild-type mice in morphology or IL-6 in the ileum. There was less ($p<0.05$) bacterial translocation after ethanol and burn injury in MLCK knockout mice than in their wild-type counterparts. Ethanol-exposed and burn-injured mice given a long MLCK specific inhibitor, membrane permeant inhibitor of MLCK (PIK), had reduced bacterial translocation than comparably treated wild-type mice not given PIK. PIK treatment did not affect intestinal morphology or ileum IL-6 levels at 6 hours after insult. Twenty-four hours after the combined insult, MLCK knockout mice had decreased intestinal morphological damage and IL-6 ($p<0.05$), but no changes in bacterial translocation as compared to similarly treated wild-type mice. At 24 hours after insult, mice treated with PIK after ethanol and burn injury had diminished IL-6 and bacterial translocation in comparison with mice not receiving PIK. These data suggest that loss of MLCK promotes restoration of intestinal morphology and barrier function, as well as reduction of intestinal inflammation, but at distinct phases after ethanol exposure and burn injury. (Supported by NIH R01AA012034 (EJK), T32 AA013527 (EJK), R01 DK068271 (JRT), Margaret A. Baima Endowment Fund for Alcohol Research and The Ralph and Marian C. Falk Medical Research Trust).

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Generation of a Macrophage-specific Mcl-1 Transgenic Mouse to Investigate Alveolar Macrophage Apoptosis in Pneumococcal Disease

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Respiratory defence against *Streptococcus pneumoniae* (Spn) infection is co-ordinated by alveolar macrophages (AM). Phagocytosis and intracellular killing of bacteria, followed by apoptosis of AM, is an important host defence mechanism. The anti-apoptotic protein Mcl-1 regulates AM viability during Spn infection. We hypothesise that Mcl-1-mediated AM apoptosis is critical for host defence against Spn. The objective of this study was to generate a macrophage-specific Mcl-1 transgenic mouse as a model to study macrophage apoptosis during pneumococcal infection. Human Mcl-1 cDNA was cloned into a pcDNA expression plasmid containing 2.9kb 5' flanking sequence and the first intron of the human CD68 gene. Transgenic mice were generated by microinjection into C57Bl/6 eggs, with founders identified by PCR and bred on a C57Bl/6 background. Macrophage-specific hMcl-1 protein expression was confirmed by Western blot. Normal macrophage development and function were assessed by bronchoalveolar and peritoneal macrophage numbers in naïve mice, and by bone marrow-derived macrophage (BMDM) F4/80 levels and phagocytic function. Alterations in apoptotic resistance were assessed by changes in nuclear morphology following UV irradiation or Spn infection. hMcl-1 protein was detected in peritoneal macraophages and BMDMs of transgenic mice but not in wild-type litter mates. Alveolar and peritoneal macrophage numbers, and BMDM F4/80 expression and phagocytic function were not altered by the hMcl-1 transgene. Over-expression of Mcl-1 in transgenic macrophages conferred increased resistance to apoptosis. Alveolar and peritoneal macrophages were less susceptible to UV irradiation, and BMDM demonstrated decreased apoptosis during an *in vitro* Spn infection. Characterisation of disease progression *in vivo* will allow us to more closely examine the role of AM apoptosis in pneumococcal disease.

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Lysosomal degradation of Staphylococcus aureus peptidoglycan is essential for the induction of macrophage inflammatory responses during infection

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Staphylococcus aureus primarily exists as a commensal bacterium in humans, though recently infections and drug resistance have been on the rise, especially in hospital settings. Even in cases of pathogenic infection, *S. aureus* induces poor protective immunity against re-infection. While the precise mechanisms preventing protective immunity are unclear, we recently demonstrated that *S. aureus* peptidoglycan (PGN) induces low levels of the important inflammatory cytokines IL-1 β and IL-18 from macrophages. *S. aureus* is able to modify its PGN, making it poorly recognized by lysozyme and thus not easily degraded by phagosomal enzymes, as a consequence the inflammatory responses of the host are reduced. Further characterization of a lysozyme-sensitive

mutant of *S. aureus* has revealed that in addition to IL-1 β and IL-18, other inflammatory responses, like TNF α and nitric oxide production, are reduced in *S. aureus* infected macrophages. These results imply that *S. aureus* has evolved a specific PGN modification that prevents the maximal innate immune response by the host, and probably also dampens the subsequent adaptive immune response, resulting in poor protective immunity after a *S. aureus* infection.

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Determination of the mechanisms by which J2 virus immortalizes murine $ human cells: a model for understanding emerging human diseases of murine virus origin and how SLC11A1 links murine virus infections with development of human leukemias

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Animal virises are known to be transmissible to humans. Typical and recent examples are the avian (H5, H7 & H9) and swine (H1N1) influenza A viruses. The J2 virus causes Lung hemorrage in mice. The J2 virus is a mouse retrovirus carrying the v-raf and v-myc oncogenes. In the presence of growth factor, this virus possesses the potential to induce onogenic transformation of adherent Bone Marrow Derived Macrophages(BMDM) and Dendritic Cells. In addititon, oncogenic transformed murine erythroid cells, as well as human lymphocytes induced by J2 virus are with reduced capacity to differentiate. Our hypotheses are that the J2 virsu hijacks intracellular and extracellular exosome-vesicle transport mechanisms for virus intergration, cell proliferation, and subsequent immortalization of cells to cause disease in both mouse and humans. Secondly that Slc11a1 levels determines the transformation proficiency of this J2 virus. There are studies in literature demonstrating the necessity of C/EBPbeta for J2 virus mediated oncogenic transformation. CEBP/beta deficient cells expressing reduced levels of prosurvival insulin like growth factor (IGF-1) needed growth factors for survival and displayed impaired tumorigenicity. Our Slc11a1 microarray data show that Slc11a1 gene disrupted MCSF stimulatyed BMDMs, expressed exceedingly significant higher CEBPB and IGF-1 transcripts at basal levels than their counterpart Slc11a1 wildtype macrophages. Futhermore, we have gene expression data showing that Slc11a1 gene disrupted macrophages has significantly higher expression of genes involved in vesicle transportation namely: vesicle tehtering, docking, fusion and export. Basal levela of AIM2, a DNA sensor was elevated inSlc11a1 gene disrupted macrophages. We have transformed Slc11a1 gene disrupted and wild-type BMDMs and BMDCs with this J2 virus. Slc11a1 gene disrupted macrophages obtained from all five mice were immortalized following supernatant transfer of J2 virus, by contrast, only 3 of six independent Slc11a1 wild type BMDMs or BMDCs were immortalized. Transformed Slc11a1 gene disrupted macrophages were less adherent to tissue culture plate than Slc11a1 wild-type macrophages. Finally, J2 virus was unable to transform human CD14 positively depleted MCSF stimulated macrophages. However, J2 viral transformation was possible for human PBMC and lymphocytes. Of note, was the fact that addition of human male AB sera to cell culture medium inhibited this transformation. Culture of cells in DMEM culture medium along with fetal bovine serum promoted J2 oncogenic transformation. All J2 virus transformed cell-lines are now stored in liquid nitrogen. Future research will focus on 1) the role of Inflammasome genes, Nucleic Acid sensing PRR, p62 sequestosome-1/interacting partners and exosomes in J2 virus oncogenic transformation of cells. 2) the molecular differences between J2 virus oncogenic transformation of murine versus human cells, especially the preference of J2 virus for human

lymphocytic transformation over myeloid cells, 3) Cellular differentiation capacity of J2 transformed murine monocytes and human lymphocytes and 4) delineate the components of human and bovine sera necessary for accelerated viral oncogenic transformation. Lastly. the necessity of SLC11A1 for human cell transformations will be determined. These should help in our understanding of mechanisms of transmission of emerging diseases due to virus transfer from animals to humans.

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The Role of CXCR3 in the Pathogenesis of Acute Intraabdominal Sepsis

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Introduction: Previous studies show that NK, NKT and $CD8^+$ T cells facilitate systemic inflammation during septic shock caused by cecal ligation and puncture (CLP). The chemokine receptor CXCR3 is expressed by those cell populations but its role in modulating lymphocyte functions during sepsis is unknown. The goal of this study was to examine the pathobiology of CXCR3 during CLP-induced septic shock.

Methods: The expression of CXCR3 by lymphocytes before and after CLP was determined using flow cytometry. CXCL9 and 10 concentrations in tissues and plasma were determined by ELISA. Pro-inflammatory cytokine production, bacterial clearance and leukocyte migration were evaluated in mice after treatment with neutralizing antibodies against CXCR3 prior to CLP.

Results: CXCL9 and CXCL10 production was induced in all tissues examined (liver>plasma>peritoneal lavage>lung) and peaked at 8 hours after CLP. An average of 45% of splenic NK cells were $CXCR3^+$ in control mice and their numbers progressively decreased between 4 and 16 hours after CLP with a concomitant increase in $CXCR3^+$ NK cells within the peritoneal cavity during the same time period. Greater than 90% of splenic NKT cells constitutively expressed CXCR3 but their numbers did not change in spleen and they were not present in the peritoneal cavity after CLP. Treatment with anti-CXCR3 24 hours prior to CLP caused depletion of significant numbers of NK, NKT and T cells in spleen and peritoneal cavity and caused significant attenuation of CLP-induced hypothermia and decreased bacterial numbers in the lung. Bacterial numbers in the peritoneal cavity and blood as well as plasma concentrations of IL-6 were not altered by anti-CXCR3 treatment.

Conclusions: CXCR3 appears to play an important role in regulating NK, but not NKT or T, cell migration into the peritoneal cavity during acute intraabdominal sepsis. Treatment with anti-CXCR3 causes depletion of $CXCR3^+$ lymphocytes and attenuates CLP-induced physiologic dysfunction.

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Differential Susceptibility of CF and Non-CF Pathogens to Antimicrobial Oxidants

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Oxidant production by neutrophils is one of the major mechanisms to combat infections. Cystic fibrosis (CF) lung disease is characterized by persistent bacterial infections, which has a unique profile of pathogens predominated by *Pseudomonas aeruginosa* (PsA) and *Staphylococcus aureus* (SA). Because of the potential deficient chloride transport to the phagosomal lumen of CF neutrophils, which is linked to deficient hypochlorous acid (HOCl) production, we predicted that the pathogens that require HOCl for efficient killing should concur with the clinical infection profile of CF. To test

the prediction, we compared the susceptibility of various CF and non-CF pathogens, including PsA, SA, *Burkholderia cepacia* (BC), *Escherichia coli* (EC) and *Klebsiella pneumoniae* (KP), to various concentrations of hydrogen peroxide (H_2O_2) or HOCl, in vitro. The results show that PsA was most resistant to H_2O_2 (LD_{50}= ~1500 μM) and HOCl (LD_{50}= ~50 μM). SA was relatively susceptible to H_2O_2 (LD50= ~100 μM) but resistant to HOCl (LD_{50} = ~35 μM). Surprisingly, KP was highly resistant to H_2O_2 (LD_{50}= ~2500-5000 μM) but was readily eradicated by low doses of HOCl (LD_{50}= ~15 μM). BC was intermediately resistant to both oxidants: H_2O_2 (LD_{50}=300-400 μM) and HOCl (LD_{50}= ~25 μM). EC displayed the least resistance to H_2O_2 (LD_{50}=200-300 μM) and HOCl (LD_{50}= ~15 μM). Further mechanistic studies demonstrate that the tested oxidants were able to breach cell membrane integrity and abolish ATP production. However, the kinetics did not fit the viability curve. These data provide an explanation to the clinical observation that PsA, an oxidant-resistant pathogen, predominates the CF lung infections. Moreover, the oxidants act on the cell membrane and energy-producing machinery, but their effects lag behind the loss of bacterial viability. This research was supported by NIH grant R01AI72327.

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TLT2 Ligation Potentiates Neutrophil Recruitment to Sites of Inflammation

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The TLT2 receptor is a member of the TREM locus, a cluster of genes encoding eight receptors in mice and humans. Of these, four are direct homologues, Trem-1, Trem-2, Trem like transcript (TLT)-1, and TLT2. TLT2 is unique among these receptors as it does not contain structural motifs that promote phosotyrosine based signal transduction and is expressed by macrophages, neutrophils, and B lymphocytes in mouse and human; thus TLT2 is the only member of the TREM family expressed by cells of both the adaptive and innate immune systems. Ligation of the TLT2 receptor on murine neutrophils results in enhanced generation of reactive oxygen species (ROS) in response to exposure to the formylated peptide FMLP. This potentiation of ROS production is similar in magnitude, but differs kinetically from the observed priming effects of LPS. Additionally, TLT2 engagement potentiates other cellular responses to FMLP, including the chemotatic response as well as degranulation. Furthermore, ligation of TLT2 enhances the migratory response of neutrophils to the murine chemokines KC, and MIP-2, their human homologue IL-8, as well as to the activated complement component C5a. The cellular responses to GM-CSF and LPS were unaltered, however, suggesting that TLT2 specifically potentiates the response of neutrophils to signals derived from G protein-coupled receptors. Administration of anti-TLT2 antibodies in mice results in an increase in the number of neutrophils that accumulate in response to croton oil, a nonspecific inflammatory mediator, supporting a role for TLT2 in enhancing neutrophil transmigration into inflamed tissue. Additionally, competitive adoptive transfer experiments demonstrate a specific enhancement of anti - TLT2 treated neutrophil accumulation in experimentally induced lung inflammation. Collectively these results demonstrate that TLT2 engagement specifically potentiates the response of neutrophils to GPCRs resulting in increased cellular responses to signals derived from these receptors including transmigration into sites of inflammation and enhanced antimicrobial activity.

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C1q Enhances Phagocytosis And Actin Polymerization Through A Pertussis Toxin Sensitive Pathway

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C1q is a member of a family of pattern recognition proteins called defense collagens, which regulate monocyte/macrophage functions including enhancement of phagocytosis and modulation of cytokine synthesis. While there has been extensive effort to identify the receptor required for defense collagen triggered enhanced phagocytosis, the receptor has remained elusive. In this study, Fc-receptor mediated phagocytosis by murine bone marrow-derived macrophages (BMDM) was enhanced with C1q, leukotriene B_4 (LTB_4) and fibronectin. LTB_4 mediated phagocytic activity occurs via BLT-1, a pertussis toxin (PTx) sensitive G α *i*-protein coupled receptor. Similar to LTB_4, C1q-, but not Fn-, mediated enhanced phagocytosis in BMDM was inhibited 58 ± 17% with PTx treatment. BLT-1 antagonist U75302 inhibited LTB_4 and not C1q-mediated phagocytosis indicating that these pathways are distinct. Moreover, we show that C1q enhanced the engulfment of the macrophage intracellular pathogen *Mycobacterium avium*, and this enhanced engulfment was also blocked by PTx. Despite the enhanced phagocytosis, there was no difference in TNF-α production from BMDM cocultured with *M. avium* in the presence of C1q when compared to control BMDM. C1q-dependent engulfment of *M. avium* was independent of LRP-1/CD91, a phagocytic receptor proposed to regulate C1q-dependent functions. Wildtype and $LRP^{-/-}$ BMDM adherent to C1q showed a 3.3 to 4 fold enhancement of phagocytosis respectively, compared to control BMDM. Pathway analysis of microarray data from BMDM treated with C1q revealed changes in gene expression associated with cytoskeletal remodeling. In support of these data, C1q, but not Fn, triggered a PTx-sensitive enhancement in actin polymerization. BMDM adherent to C1q showed a 2.8 fold increase in phalloidin labeling compared to controls. Taken together, these studies demonstrate that C1q-mediated enhanced phagocytosis and cytoskeletal alterations are mediated by a PTx sensitive pathway, and should contribute towards identification of the receptor(s) involved in defense collagen mediated regulation of monocyte/macrophage immune responses.

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Synergic innate immune recognition of Coxsackievirus B5 by RIG-I and MDA5

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Coxsackievirus B5 (CBV5) is a positive sense, single-stranded RNA virus belonging to the *Picornaviridae* family. It can cause many diseases, including myocarditis, dilated cardiomyopathy, aseptic meningitis, and pancreatitis. Its structure and cell cycle is typical of a picornavirus. Upon infection, the viral RNA is detected by retinoic acid inducible gene I (RIG-I)-like helicases (RLHs) and Toll-like receptors (TLRs). The RLHs include RIG-I and melanoma differentiation associated gene 5 (MDA5), as well as Lgp2. Once viral RNA has been detected, the RLHs trigger a signalling cascade, via CARD-CARD interactions with interferon-β promoter stimulator 1 (IPS-1, also known as MAVS, VISA, and Cardif), which leads to the activation of the transcription factor NF-κB and pro-inflammatory cytokines, to mediate induction of the innate immune response. RIG-I and MDA5 preferentially detect different viral pathogen-associated molecular patterns. Previous results suggest MDA5 is critical for picornaviridae detection, whilst RIG-I is essential for flaviviridae, paramyxoviridae,

orthomyxoviridae, and rhabdoviridae detection. Results presented here suggest that RIG-I may play a more important role in CBV5 detection than previously thought. Confocal microscopy images show increased co-localisation of RIG-I and MDA5 with IPS-1 upon stimulation with CBV5 for 1, 2, 4, and 6 hours in Cardiac cells, corresponding to the expression levels obtained using indirect immunofluorescence coupled with flow cytometry. This suggests RIG-I and MDA5 work in tandem to detect CBV5 and elicit a downstream immune response.

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A Method for cryopreservation of mouse bone marrow cells for further generation of bone marrow-derived macrophages

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The broad use of transgenic and gene target mice established the bone marrow-derived macrophages (BMDM) as an important mammalian host cell for the investigation of the immune functions of macrophages. Over the last decade, it was extensively investigated how to freeze and store viable hematopoietic human cells, however, there is no information regarding to generation of murine BMDM from frozen murine bone marrow (BM) cells. We establish a highly efficient protocol to freeze murine BM cells and further generate BMDM. We compared BMDM obtained from fresh or frozen BM cells and found that both are similarly able to trigger the expression of the stimulatory molecule MHC-II and the co-stimulatory molecule CD86 in response to inflammatory stimulus, such as LPS or infection with the intracellular bacteria *Legionella pneumophila*. Accordingly, BMDM obtained from fresh or frozen BM cells equally restrict the multiplication of the protozoan parasite *Leishmania amazonensis* and the bacteria *L. pneumophila*. Finally, we demonstrated that cryopreserved murine BM cells maintain their ability for BMDM differentiation for more than 6 years. Overall, the method described allows ready and easy generation of BMDM from a stock of frozen BM cells.

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Identification of macrophage transcriptional responses in response to Pseudomonas Domestic Substance List microbes

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In vitro testing of Domestic Substance list (DSL) microorganisms, in support of the Canadian Environmental Protection Act (1999) can be complemented by genomic based exposure tests in order to provide additional quantitative and mechanistic data. In this presentation, an overview of mouse macrophage responses to DSL Pseudomonad strains is shown at the levels of gene transcription of recognition and immune response genes. Mouse macrophage like cells (J774A.1) were exposed to strains including: *P. aeruginosa* (Pa), *P. fluorescens* (Pf) and *P. stutzeri* (Ps). Receptor and immune gene response from exposed macrophage and control (mock-exposed) cells was quantified by real time reverse transcription PCR (RT-PCR). A screen for ASE in immune related genes, was carried out to compare alternatively spliced regions in RNA samples from control (non-exposed cells) and specific Pa strain exposures. All microbial strains caused significant changes in macrophage pro-inflammatory genes, toll like receptors and genes in the NFkB pathway. However, changes in gene expression in response to Pa strains were either unique, or greater in magnitude than for tested Pf and Ps strains. A preliminary ASE screen of immune related genes has yielded two candidates that show splice events in particular microbial exposures. Receptor and immune gene expression profiling has the potential to provide differences in macrophage cell perturbation to

related bacteria and these differences appear to correlate to other data collected in our group. The observed splice events are now being validated in other Pa exposures and additional gene expression studies. Together, these studies show potential in identification of particular transcriptional differences in the effects of microbes on macrophage cells.

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Identification of Functional miRNAs in Macrophages by Analysis of Minor Strand miRNA Expression

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The intracellular protozoan parasites of the *Leishmania* spp. infect macrophages and cause a broad spectrum of diseases in humans, some of which are life-threatening. Currently, no safe vaccines are available to prevent leishmaniasis and therapies are complicated by emerging resistance and harmful side effects. We are evaluating the importance of microRNAs in regulating macrophage activities, based on the hypothesis that small RNAs could become novel therapeutic agents for leishmaniasis. MicroRNAs (miRNAs) are a class of small, non-coding RNAs, some of which are known to alter macrophage function. Using qRT-PCR arrays, we identified miRNAs whose expression in macrophages was significantly altered by stimuli that polarize macrophages toward different phenotypes. Several miRNAs were altered significantly by one or more macrophage conditions: classically activated (M1), alternatively activated (M2a), type II activated (M2b), and deactivated by TGF-beta treatment (M2c). Fold changes in miRNA abundance were small in polarized primary macrophages, and the fold change in abundance of the cognate minor strand miRNA (miRNA*) often exceeded those of the miRNA early after polarization. miRNAs, but not miRNAs*, were functional in HEK293 transfection studies. Ectopic introduction of miRNA mimics by transfection into macrophages augmented the abundance of selected miRNAs. Functional analyses revealed that some candidate miRNA mimics altered macrophage chemokine/cytokine expression and also altered macrophage responses to LPS. Target prediction software, pathway analyses, and luciferase reporter assays are being utilized to validate the miRNA targets. Toward the goal of identifying miRNAs that potentiate macrophage leishmanicidal activity, we have generated luciferase- and mCherry-expressing *Leishmania* and developed novel screening assays to track parasite burden in transfected macrophages. We hypothesize that ectopic expression of miRNAs that lead to macrophage polarization could alter parasite burden in *Leishmania*-infected macrophages.

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Bacterial Motility Facilitates Leukocyte Phagocytosis of Pseudomonas aeruginosa

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Objective and Methods: *Pseudomonas aeruginosa* is a pathogenic Gram-negative bacterium that causes severe opportunistic infections in immunocompromised individuals; severity of infection with *P. aeruginosa* positively correlates with poor prognosis in cystic fibrosis patients. Establishment of chronic infection by this pathogen is associated with the downregulation of flagellar gene expression and phenotypic loss of *P. aeruginosa* motility. Using multiple bacterial and mammalian genetic approaches, we investigate why loss of bacterial motility is the critical factor in the development of resistance to phagocytosis by *P. aeruginosa.*

Results : Here we demonstrate that loss of bacterial motility, not the loss of the flagellum *per se*, is the critical factor in the development of resistance to phagocytosis by *P. aeruginosa*. Isogenic *P. aeruginosa* mutants deficient in flagellar function, but retaining an intact flagellum, are highly resistant to phagocytosis by both murine and human phagocytic cells at levels comparable to those of flagellum-deficient mutants. As a logical extension of these findings we therefore tested the hypothesis that phagocytosis is proportional to bacterial motility. Four partially-redundant flagellar stator proteins provide rotational torque for *P. aeruginosa* swimming motility. We show that wild-type levels of phagocytic susceptibility require expression of all four stator genes, and demonstrate for the first time that partial losses of the stator complex results in intermediate degrees of phagocytic resistance. Only the complete loss of all four stator proteins confers resistance equal to that of flagellum-deficient mutants. Intriguingly, bacterial-phagocyte binding only minimally affects *P. aeruginosa* phagocytosis, supported by the observation that the non-swimming strains exhibit resistance to engulfment even after phagocyte contact.

Conclusions: These findings provide a mechanistic explanation for the prevalence of non-motile *P. aeruginosa*clinicalisolates and for how this phenotype confers upon the bacteria an advantage in the context of immune evasion. Funding: NIH RO1 AI067405, CF Foundation STANTO07R0, and NIH Training Grants T32 AI07363 and GM008704.

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The critical role of Caspase-1/IL-1beta and inflammasome in Chlamydophila pneumoniae Lung infection

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We have previously shown that TLR/MyD88 signaling molecules are essential in recognition and host responses against *Chlamydophila pneumoniae* (CP) infection leading to clearance of bacteria from the lung. **Goals**: Since MyD88 mediates signaling from both TLRs and IL-1R, here we investigated the specific role of IL-1β secretion and the activation of the inflammasome in host responses against CPlung infection. **Methods:** Caspase-1 (Casp1)-/- mice were intratracheally infected with CP, and bacterial clearance from the lungs, pulmonary inflammation, as well as mortality were documented. **Results**: Subleathal dose of CP infection lead to significantly delayed bacterial clearance from the lungs, increased chronic lung inflammation, and increased mortality in Casp1-/- mice. IL-1β release in BALF was impaired in Casp1-/- mice. IL-6 and IFNγ production in BALF and lung homogenates was delayed in Casp1-/- mice compared to WT mice and Casp1-/- alveolar macrophages displayed defective iNOS production, a critical bactericidal component against CP. Blocking the IL-1β signaling by injections of IL-1 receptor antagonist (IL-1RA) into WT mice also resulted in impaired bacterial clearance and increased mortality similar to the Casp1-/- mice. Moreover, injection of recombinant IL-1β into Casp1-/- mice rescued these mice from CP infection by restoring bacterial clearance and preventing mortality. CP-induced Caspase-1 activation/IL-1β release in BM-derived macrophages were NALP3-, and ASC-dependent. Of interest CP-induced NALP3 activation was independent of the three proposed models of inflammasome activation (ROS, K+ efflux or Lysosomal degradation). **Conclusion:** Our findings demonstrated CP-induced Casp1 activation and IL-1β secretion play a critical role in host defenses against CP lung infection. (This study is supported by NIH RO1 AI067995MA).

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Activated NK Cells Control Intracellular Leishmania Parasites without Lysis of Infected Host Cells

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Introduction: The innate immune response against the intracellular protozoan parasite *Leishmania (L.) infantum*, an agent of visceral leishmaniasis, is characterized by the rapid activation of natural killer (NK) cells. Activated NK cells exert cytotoxic activity and release interferon (IFN)- γ, which are thought to cause parasite killing via host cell (and parasite) lysis or via IFN- γ -dependent stimulation of macrophages for the production of leishmanicidal nitric oxide, respectively. Here, we investigated to what extent both NK cell effector functions contribute to the control of *Leishmania* parasites.
Methods: The cytotoxic activity of splenic NK cells against different target cells was determined in vitro by a ^{51}Cr release assay. In vivo, the cytolysis of *Leishmania*-infected macrophages that were labelled by CFSE was analysed after transfer into *Leishmania*-infected recipient mice that exhibited NK cell activity. The ability of NK cells to trigger macrophage-mediated killing of *Leishmania* was investigated in vitro by 72 h co-culture of both cell types and microscopic analysis of infection rates and intracellular parasite numbers.
Results: Although NK cells from *L. infantum*-infected mice showed high cytolytic activity against tumor target cells, they failed to lyse infected host cells such as macrophages, dendritic cells or fibroblasts in vitro. *Leishmania* -infected wildtype macrophages that were transferred into *L. infantum*-infected recipient mice, did not become a target of activated NK cells, whereas transferred ß2m-/- macrophages were rapidly eliminated by the recipient NK cells. When infected macrophages were co-cultured with IFN- γ producing activated NK cells purified from infected mice by cell-sorting, the number of intracellular parasites clearly decreased.
Conclusions: Activated NK cells do not directly kill infected host cells, but are still able to induce a decrease of the parasite load inside infected macrophages in a co-culture system. Thus, it is not the cytolytic potential, but rather the NK cell-derived IFN- γ that contributes to the control of *Leishmania* parasites in host cells.

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Mycobacterium tuberculosis induces hepcidin production by epithelial cells and dendritic cells

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Hepcidin is a regulator of iron homeostasis that also functions as an antimicrobial peptide. Previously, we demonstrated that hepcidin possessed antimicrobial activity against *Mycobacterium tuberculosisin vitro*, and that macrophages produced hepcidin by the synergistic interaction of IFN-γ and *M. tuberculosis* infection. Apart from macrophages, interactions of the bacilli with epithelial cells and dendritic cells play a role in immunity to *M. tuberculosis*. In the present study, we report that *M. tuberculosis* and its components stimulate the production of hepcidin by epithelial cells and dendritic cells. In human alveolar A549 epithelial cells, IFN-γ and lipoglycans of *M. tuberculosis* were better inducers of hepcidin than subcellular fractions of the bacillus. In contrast, mouse dendritic cells produced higher levels of hepcidin in response to subcellular fractions and culture filtrate proteins, but not lipoglycans of *M. tuberculosis*. Moreover, TLR2 and TLR4 agonists, but not TLR9 agonists or IL-1α, IL-6 and TNF-α induced hepcidin in mouse dendritic cells. Flow cytometric evaluation of human peripheral blood mononuclear cells demonstrated that killed *M. tuberculosis* or live *M. bovis* BCG induced

hepcidin production by CD11c+ myeloid dendritic cells. Taken together, our results suggest that the induction of hepcidin by cells that play key roles in the generation of a protective immune response to *M. tuberculosis* is important to the pathogenesis of tuberculosis.

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MMP-9 mediated neutrophil migration – Broad spectrum immune based therapeutic target in respiratory tularemia

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MMP-9 activity has been associated with host susceptibility to pulmonary infection with *Francisella tularensis*. MMP-9 activity enhances infiltration of *F. tularensis*-infected lungs by neutrophils resulting in increased morbidity and mortality. This notion is supported by the greater susceptibility, higher bacterial burden, and increased tissue pathology seen in wild type mice compared with those deficient for MMP-9. Neutrophils likely contribute to the greater destruction of the lung parenchyma in MMP-$9^{+/+}$ mice. Collagen-derived peptides such as PGP (N-acetyl-proline-glycine-proline) which act through CXCR1 and CXCR2 have been reported to be chemotactic for neutrophils. We hypothesized that use of small molecule antagonists that selectively block PGP and CXCL-8 (which also acts through CXCR1/2) may reduce neutrophil infiltration and thus lessen the destructive effects of these cells. In our mouse airway model we have seen that PGP induces neutrophil migration in a time dependent manner. We have used an inhibitor, RTR (L- arginine-threonine-arginine), which blocks both the PGP and CXCL pathways and thus blunts the neutrophil influx. RTR alone is incapable of recruiting neutrophils as was observed in a dose dependent assay. However, RTR does prevent migration of neutrophils induced by PGP. We intend to use this inhibitor to reduce neutrophil recruitment in *F. tularensis*-infected MMP-$9^{+/+}$ mice to levels observed in infected MMP-$9^{-/-}$ mice (potentially prolonging their survival). This strategy may provide a novel adjunct to antibiotic-based therapeutic intervention in respiratory tularemia.

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Pathogen Effector Protein Screening in Yeast (PEPSY) Identifies Rv3812 as a Candidate Mycobacterium tuberculosis Virulence Factor

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One third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb),leading to three million deaths per year. Mtb infects macrophages and has developed mechanisms to avoid being killed by these effector cells. A key strategy is its ability to inhibit phagosome maturation, but how Mtb disrupts vesicle trafficking in macrophages is not fully understood. To identify potential Mtb effectors involved in pathogenesis and inhibition of phagosome maturation, we carried out a genetic screen in yeast for proteins that disrupt yeast vacuolar trafficking. Many bacterial effectors involved in mammalian infection retain their function in yeast, and the yeast vacuole protein-sorting pathway (VPS) is well characterized, with mammalian VPS orthologs regulating vesicular traffic in the endosomal system. Thus, pathogen effectors that interfere with the yeast VPS pathway may also disrupt mammalian endosomal trafficking.

We screened an Mtb H37Rv genomic library using PEPSY and in initial screens identified four positive hits, corresponding to ORFs from Rv0425c (an ATPase), Rv0900 (a membrane protein), Rv1268c (a secreted protein), and Rv3812 (a PE_-PGRS protein). Notably, Rv3812 has been linked to virulence as the *M. marinum* knockout of this gene is attenuated in macrophages and in mice. These results suggest that PEPSY can identify Mtb proteins that target the yeast VPS pathway. These proteins are candidates for targeting the endosomal system in macrophages which may in turn disrupt phagosome maturation. In order to study the effects of Rv3812 on macrophage function, an

M. smegmatis construct was made which allowed the expression of histidine tagged Rv3812 protein upon growth in media containing acetamide. Subcellular fractionation of this *M. smegmatis* construct indicated the presence of Rv3812 in the cell wall fraction. J774A cells were infected with Rv3812 expressing *M. smegmatis* grown under inducing and noninducing conditions, and the response to infection was assessed. J774A infected with *M. smegmatis* expressing Rv3812 show reduced expression of inducible nitric oxide synthase (iNOS) when compared to cells infected with control *M. smegmatis.* Work is currently underway to determine the mechanism by which Rv3812 interferes with iNOS expression, as well as to assess the effect of this protein on macrophage phagosome maturation.

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Silencing of Socs Genes Increases Cardiac Inflammation and Reduces Cardiac Function after Hemorrhagic Shock (HEM)

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Pro-inflammatory cytokines play an important role in the pathogenesis of cardiac dysfunction. Suppressor of cytokine signaling (SOCS) proteins have been identified as feedback regulators of cytokine-induced JAK/STAT signaling. Studies have shown that SOCS-1 and -3 mRNA are upregulated in the heart after injury. However, the contribution of SOCS-1 and/or -3 protein to heart function after HEM is still unclear. The aim of this study was to determine the involvement of SOCS-1 and/or -3 in the regulation of cytokine-mediated cardiac injury/inflammation following shock. Male C57BL/6 mice were subjected to sham or HEM (90 min at 30±5mmHg and resuscitated with Ringers, 4X shed blood vol.) with control or SOCS siRNA treatment in vivo and the hearts were harvested at various times post-surgery. SOCS-1, -3 or phosphorylated STAT-3 proteins were detected by immunoblotting and gene expression by RT-PCR. Cytokine levels were assessed by cytometric bead array or ELISA. Myeloperoxidase activity was measured enzymatically. Organ blood flow and cardiac output were determined using fluorescent microspheres. Our data show that SOCS-1 and –3 gene expressions were up-regulated after HEM compared to shams. Two hrs after HEM, which was the peak time for SOCS gene expression, there was a general increase in IL-12, TNF- α , MCP-1, IL-6 and IL-10 in the heart. However, these cytokines in the heart were further increased when SOCS-1 or –3 was knock-down by siRNA. Similar results were observed in the plasma cytokine levels after HEM. Heart blood flow and cardiac output were decreased in siRNA treated shock mice when compared with control mice. An increase in STAT-3 phosphorylation of the heart was correlated with the higher IL-10 & IL-6 levels and myeloperoxidase activity in mice treated with SOCS-1 siRNA. Taken together, these results suggest that the SOCS proteins potentially play an important role in regulating cytokine activation and heart function after shock.

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Regulation of MicroRNAs in Mycobacterium tuberculosis-infected Macrophages

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MicroRNAs (miRNAs) are a class of small (about 22nucleotides) endogenous RNA molecules that negatively regulate gene

expression by translational repression. miRNA block translation and/or accelerate mRNA decay by binding to complementary sequences in the mRNA. There is increasing evidence in favor of the view that miRNAs play an important role in innate immunity, for example by limiting the inflammatory response. There has so far been no attempt to understand the miRNA network in the context of tuberculosis infection. We have used murine macrophages infected with *Mycobacterium tuberculosis* as our model and made an analysis of the expression of a group of 89 miRNAs. Our studies provide evidence of the prominent upregulation of miR-155 in *M. tuberculosis*-infected macrophages. miR-155 is one of the best studied regulators of the innate immune response. In addition we have also observed the up- or down-regulation of a number of other miRNAs. One of these is miR-146. These miRNAs influence the innate immune response to *M. tuberculosis* infection.

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Human TLR10 is a TLR2 family member with a unique innate immune function.
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TLR10 is the only human TLR family member without a defined agonist or function. Phylogenetic analysis reveals that TLR10 is most related to TLR1 and TLR6, both of which mediate immune responses to a variety of microbial and fungal components in cooperation with TLR2. The generation and analysis of chimeric receptors containing the extracellular recognition domain of TLR10 and the intracellular signaling domain of TLR1, revealed that TLR10 senses triacylated lipopeptides and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6. TLR10 requires TLR2 for innate immune recognition, and these receptors colocalize in the phagosome and physically interact in an agonist-dependent fashion. Computational modeling and mutational analysis of TLR10 showed preservation of the essential TLR2 dimer interface and lipopeptide-binding channel found in TLR1. Coimmunoprecipitation experiments indicate that, similar to TLR2/1, TLR2/10 complexes recruit the proximal adaptor MyD88 to the activated receptor complex. However, TLR10, alone or in cooperation with TLR2, fails to activate typical TLR-induced signaling, including NF-kappaB-, IL-8-, or IFN-beta-driven reporters. We conclude that human TLR10 cooperates with TLR2 in the sensing of microbes and fungi but possesses a signaling function distinct from that of other TLR2 subfamily members. Recent results, including findings from TLR10 transgenic mice, suggest that TLR10 functions as an active suppressor of TLR2 signaling.

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The activation of the phosphatidyl-inositol-3 kinase pathway in response to the *Burkholderia cepacia complex* infection
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Background: Cystic fibrosis (CF) is the most common fatal inherited disease in North America and bacterial infection is the leading cause of death in these patients. Infections with the *Burkholderia cepacia* complex (*Bcc*) can result in rapid decline in lung function and death. Whithin the species of the *Bcc*, *B. cenocepacia* (*Bc*) causes more severe infections than *B. multivorans* (*Bm*), however the mechanism behind this difference in virulence remains undetermined. We investigated the signaling events, specifically in the phosphoinositide3-kinase (PI3K) signaling pathway after bacterial-host interaction to determine if measures of cellular activation are associated with the differential virulence and persistence of these bacteria in infected host cells in CF patients. **Methods:** CF bronchial epithelial cell line (IB3-1), THP-1 derived macrophage (MΦs) and human monocyte-derived MΦs were infected with *Bc* and *Bm*. Lysates from infected and uninfected cells were analyzed by Western blot or by immunoprecipitation using the appropriate antibodies. **Results:** The activation of the PI3K pathway occurred in IB3-1, in THP-1 derived MΦs and in human monocyte-derived MΦs after

infection with live *Bc*. PI3K was not activated by *Bc*-LPS in IB3-1 cells but only with live *Bc*,andthisactivation was seen up to 3 hours after bacterial challenge. PI3K activation was faster in *Bm* (5 min) than Bc (30 min) after infection in MΦs. Activation of PI3K was abolished after infection with PI3K inhibitors. The release of TNF-α in MΦs infected with *Bc* and *Bm* in the presence of PI3K inhibitor was decreased. **Conclusion** :The PI3K signaling pathway is activated in response to the *Bcc* in both MΦs and epithelial cells. *Bm* showed a more rapid phosphorylation after infection as compare to *Bc* in MΦs. Current studies are in progress to correlate the rate of PI3K phosphorylation with cell death via apoptosis or necrosis assays. This work was supported by a grant from the Canadian Cystic Fibrosis Foundation (to D.P.S.).

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NMR Analysis of Purified Lipid A in a Membrane Mimetic

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A new HPLC- protocol for the isolation of highly purified lipid A (LA) from *Escherichia coli* mutants F515 and WBB 01 LPS resulted in numerous LA sub-fractions, varying in their acylation and phosphorylation pattern. Besides *E. coli*-type LA (compound 506, LA-6), we identified hepta-acylated LA (LA-7) with 14:0[3-*O*(16:0)] at 2' and penta-acylated LA [LA-5(1)] lacking the myristic acid (14:0). A second penta-acylated LA [LA-5(2)], carrying (2*E*)-14:1 at position 3" was also found in one mutant (F515). Moreover, we identified a tetra-acylated LA (LA-4), lacking the acyloxyacyl residue 14:0[3-*O*(14:0)] at 3". Finally, LA-2 with two amide linked ß-hydroxylated myristic acids [14:0(3-OH)] and LA-3 with 14:0[3-*O*(12:0)] in amide linkage at 2", represented minor LA part structures.

Previously we demonstrated NMR analysis of LA-5(1) and LA-6 in water (D HPC micelles) with ^{13}C,^{15}N-labeled LA. Here we describe for the first time high-field NMR analysis (700 MHz) of natural abundance LA, showing well resolved signals in all 1D and 2D ^{1}H spectra, down to a nM sensitivity. The resolution was high enough to determine positions and configurations of all fatty acids unequivocally. The acylation pattern is known to significantly influence the biological (endotoxic) activity of LA, thus determining its agonistic and antagonistic activities. The resolution of the ^{1}H spectra appeared high, allowing to determine homonuclear ($^{3}J_{Hn,Hn+1}$) as well as heteronuclear (e.g. $^{3}J_{H,P}$) coupling constants, suitable for a refined conformational analysis. In addition, nuclear Overhauser enhancement (NOE) spectroscopy allowed conformational analysis of all LA substructures. We found a similar conformation of the LA backbone in LA-6 and LA-4 when solubilized in D HPC micelles, as it was described from x-ray analysis, being highly twisted when bound to the natural receptor MD-2.

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Incorporation of Bile Acids into Erwinia carotovora Lipid A Aggregates and Influence on Their Biological Activity and Phase Behaviors

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The neutralization of endotoxin structures such as the active 'endotoxic principle' lipid A by suitable compounds has been shown to be a key step in the treatment of infectious diseases, in particular in the case of Gram-negative bacteria which frequently may lead to the septic shock syndrome. Cholesterol related chemicals called bile acids are known to act as digesting reagents. Aggregated biological compounds are disintegrated by them. Here, the interaction of bile acids, with hexaacyl lipid A isolated from lipopolysaccharide of *Erwinia carotovora*, was investigated. By using Fourier-transform infrared spectroscopy, the gel to liquid crystalline phase transition of the acyl chains of lipid A and the conformation of their phosphate groups due to bile acids binding was characterized. The electrophoretic mobility of lipid A aggregates under the influence of the bile acids was studied to determine the Zeta potential, and small-angle X-ray scattering was applied for the elucidation of the aggregate structures. The lipid A-induced cytokine production in human mononuclear cells shows that the tumor necrosis factor-alpha production differs with characteristic changes of the biophysical parameters. These are stronger expressed for cholic acid, which apparently is connected with its carboxylic acid moiety, leading to a stronger amphiphilicity necessary to rearrange the lipid A aggregates.

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Deactivation Of The Lps Antagonist Eritoran (E5564) By Hdl-Associated Apolipoproteins

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Background*:* Severe sepsis is a complex and life-threatening immune response to infection believed to be triggered by bacterial endotoxin (also called lipopolysaccharide or LPS). Lipid A is the active moiety of LPS that exerts its effects through interaction with toll-like receptor 4 and MD-2 (TLR4/MD2) triggering a signaling cascade that results in the "cytokine storm" of pro-inflammatory cytokines such as tumour necrosis-α (TNF-α) and interleukin-1 (IL-1). Eritoran is a synthetic lipid A analogue of a nontoxic bacterium *Rhodobacter sphaeroides* and a powerful TLR4/MD-2 antagonist. However, following intravenous administration, eritoran undergoes a time-dependent deactivation as a consequence of binding to high-density lipoproteins (HDL) in blood. At present, there remains much uncertainty surrounding the site of eritoran association with HDL and therefore the objective of this study was to determine if HDL-associated apolipoproteins inhibit eritoran's ability to block LPS-induced TNF-α release from whole blood. **Methods*:*** We assessed eritoran activity as an inhibitor of LPS-mediated TNF-α release in human whole blood in the presence of free or reconstituted HDL (rHDL) containing apolipoproteins including a combination rHDL containing each of all apolipoproteins found on native HDL and septic HDL. **Results:** Differences exist in the effect on drug activity when apolipoproteins are free versus lipid-bound and the major apolipoproteins in both the healthy (apo A1) and septic state (serum amyloid A or SAA), greatly reduce antagonistic activity of eritoran. Overall, A1 and SAA are better at deactivating eritoran than the more minor apolipoproteins A2 and C1. 'Normal' rHDL (NrHDL), in which a reconstituted particle was produced containing apolipoproteins A1, A2, C1, C2, C3, and E in the molar ratio they would be found associated with a healthy HDL, deactivates eritoran to a similar degree as apo A1 alone in rHDL. This infers that apo A1 likely accounts for the deactivating effect observed with NrHDL. **Conclusions*:*** Apolipoproteins are likely factors in eritoran deactivation by HDL. Apo A1 and SAA should be of particular focus as they are the major apolipoproteins found on HDL in both the healthy and septic state, respectively, and were observed to greatly reduce eritoran activity. Further evaluation of the physical association between apolipoproteins and eritoran and the mechanisms by which apolipoproteins inactivate

eritoran, particularly binding mechanisms, should be explored. **Acknowledgements:** Funding provided by Eisai Inc. and Canadian Institutes of Health Research

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Lipopolysaccharide Induces Raft Domain Expansion in Membrane Composed of a Phospholipid-Cholesterol-Sphingomyelin Ternary System

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The molecular behavior and interaction of Re-type lipopolysaccharide (ReLPS) and phospholipids were investigated in two different types of model membrane systems, a pure phospholipid membrane consisting of 1,2-dielaidoyl-s*n*-glycero-3-phosphoethanolamine (DEPE) and a raft-forming membrane composed of equimolar DEPE, sphingomyelin (SM), and cholesterol (Chol) by solid-state NMR spectroscopy. A remarkable influence of ReLPS on the property of lipid bilayer was found by analyzing the ^{13}C-NMR spectra. Namely, while both liquid-ordered (L_o) and liquid-disordered (L_d) phases co-exist in DEPE/SM/Chol, only the L_o phase is present in DEPE/SM/Chol/ReLPS. This clearly indicates that ReLPS induces expansion of the raft area in the raft-forming membrane. This ability of ReLPS becomes more confident by the fact that the acyl chains of ReLPS adopt the *trans-gauche* conformation in DEPE/ReLPS and *all-trans* conformation in DEPE/SM/Chol/ReLPS as indicated from the chemical shift values of ReLPS $(CH_2)_n$ peaks. The ^{1}H spin-lattice relaxation times in the rotating frame $T_{1\rho}{}^H$ in the two different membranes, DEPE/ReLPS and DEPE/SM/Chol/ReLPS, indicate that the motion of DEPE is affected by the presence of ReLPS, Chol, and SM, and much faster than that of ReLPS in both membranes. The ReLPS in the raft-forming membrane, in particular, accelerated the movement of DEPE. By contrast, the motion of ReLPS in the two membrane systems is almost the same. **Thus, this study shows the possibilities that LPS acts autocatalytically in the animal cell membrane during innate immune recognition by inducing the expansion of raft region and the** rapid motion of membranes to favor molecular interactions.

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Structural and Biological Re-investigation of Synthetic and Bacterial Lipoteichoic Acid from *Staphylococcus aureus*

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Based on structural and biological similarities, lipoteichoic acid (LTA) has also been considered as the Gram-positive counterpart of Gram-negative lipopolysaccharide (LPS). The biological activity of LTA in *Staphylococcus aureus* is rather low compared to LPS. Its pro-inflammatory potency has been attributed to the degree and nature (D - or L -) of alanylation in the hydrophilic repeating units (Gro-*P*)$_n$. By contrast, the endotoxic activity of LPS is well known to reside in the lipophilic part (lipid A). For us it appeared unlikely that both glycolipids act in a similar way with respect to their structure-function relationship.

Therefore, we carefully re-investigated the structure of *S. aureus* LTA. ESI FT-MS identified a polymerization degree in the (Gro-*P*)$_n$ shorter (n ~ 10-20) than previously described (45-50). In addition we could show that the degree of D -alanylation in this LTA has no influence on its pro-inflammatory potency. NMR analysis of natural LTA in small DHPC-

micelles gave well-resolved signals, allowing structural and conformational analysis of the gentiobiose adjacent to the in the lipid anchor (diacylglycerol, DAG) in intact LTA.
We have also probed the induction of cytokines in human mononuclear cells (hMNC) and in a whole blood assay (WBA) after stimulation with *S. aureus* LTA and with synthetic LTA. Both preparations induced IL-6 and IL-8 release in h MNC and WBA. *S. aureus* LTA preparations, alanylated or not, showed comparable TLR2 activity in transfected HEK293-TLR2 cells. By contrast, this TLR2-activity was lacking in synthetic LTA, indicating contaminating lipoprotein in the "highly purified" bacterial LTA preparations. All LTA-preparations showed no activity in HEK293-TLR4/MD2/CD14, thus excluding the contribution of contaminating LPS.
In summary, inflammatory activity of LTA is not mediated by TLR2 or TLR4, but by other, so far unidentified receptor(s) most likely of the lectin pathway in the complement system.
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Lipid A characterisation of Pasteurellaceae isolates using MALDI-TOF

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Members of the Pasteurellaceae group of Gram-negative bacteria are major causes of respiratory and systemic disease in a range of domestic animals worldwide. In common with all Gram-negative bacteria, lipopolysaccharide (LPS) is a major component of its outer membrane. The lipid A fraction of LPS is poorly immunogenic but is the primary agent of the molecule's toxic effect on the host. The number and length of the acylated fatty acids attached to and the degree of phosphorylation of the di-glucosamine molecule have major effects on virulence. Lipid A with a maximum of six fatty acyl chains is highly reactive and can protect bacteria from antimicrobial peptides present in mucosal secretions. Therefore it is important to understand the lipid A composition of different species and isolates with the Pasteurellaceae in order to understand better its contribution to disease. The composition of lipid A is considered to be highly conserved within species, although the common means of classifying the somatic antigens of Gram-negative bacteria relies on antibodies and lacks the refinement and power of modern molecular methods. Improved techniques to characterise and differentiate isolates from different regions, animal species and disease states may enable us to direct more effective methods of control. The object of the study was to characterise the lipid A of nasal isolates of *Mannheimia haemolytica* and *Pasteurella multocida* using MALDI-TOF and investigate whether there was any link with geographical or animal origin, bacterial serotype or group.These isolates were subject also to multi-locus sequence typing (MLST) and results assessed to see if there were epidemiological links between these characterisations that may prove useful in differentiating virulence or different disease or host types in order to enable directed and specific methods of control to reduce associated welfare and economic problems. LPS was extracted using hot water phenol and lipid A released by 3h hydrolysis with 0.1M sodium acetate (pH 4.4) containing 1% SDS at 100°C. SDS was removed using 2M HCl:EtOH (1:100 v/v), the pelleted sample reconstituted in chloroform: methanol (2:1 v/v) and analysed by MALDI-ToF (Bruker Ultraflex II) in negative ion mode using 6-Aza-2-thiothymine matrix. Mass spectra of intensity against m/z were produced by scanning the 450-4500 Daltons region. For MLST analysis, bacterial DNA lysates were used to amplify loci from 7 housekeeping genes, using primers and conditions as per the MLST (RIRDC) scheme (http://pubmlst.org/pmultocida_rirdc/) .
Twenty-five *M. haemolytica* and 75 *P. multocida* commensal and disease isolates from nasal swabs of cattle, sheep and pig in 6 different geographical regions were analysed. The results showed that, for *M. haemolytica*, lipid A characterisation was uniform in some geographical regions but variable in others and for *P. multocida* that there were consistent differences in lipid A profiles for isolates of

different capsule type. MLST analysis showed clonality within cattle isolates, with 105/129 belonging to clonal complex (CC) 13 (sharing 6/7 alleles) that originated from UK, USA and France over different time periods (1984-2008) and showed varied pathogenicity. Most STs were specific to host of origin, with exceptions for ST13 (40 bovine and 7 porcine isolates) and ST 132 (3 ovine and 1 bovine isolates). The work showed also a close correlation between ST and LPS core banding patterns on silver stained SDS-PAGE gels of phenol-extracted LPS.

We conclude that elucidation of the lipid A structure of LPS may be used to group isolates according to molecular epidemiological profiles and provide insights into the characteristics of key pathogenic determinants of *M. haemolytica* and *P. multocida*.

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Phosphoryl Moieties of Lipid A from Neisseria meningitidis and N. gonorrhoeae Lipooligosaccharides Play an Important Role in the Differential Activation of both MyD88- and TRIF-Dependent TLR4/MD-2 Signaling Pathways

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We have previously shown that the lipooligosaccharide (LOS) from *Neisseria meningitidis* and *N.gonorrhoeae* engages the innate immune receptor TLR4/MD-2 complex. In this study, we report that LOS from different meningococcal and gonococcal strains differentially activates NF-κB through TLR4/MD-2, and that the relative activation can be correlated with ion abundances in MALDI-TOF mass spectrometry that are indicative of the number of phosphoryl substituents on the lipid A (LA) component of the LOS. The LOS from three of the strains, meningococcal strain 89I and gonococcal strains 1291 and GC56, representing high, intermediate and low potency NF-κB activation potential, respectively, differentially activated cytokine expression through the TLR4/MD-2 pathway in monocytes. In addition to induction of typical inflammatory cytokines such as TNF-α, IL-1β, and IL-6, MIP-1α and MIP-1β also were significantly higher in cells treated with 89I LOS which had the most phosphoryl substitutions on the LA compared to 1291 and GC56. We found that LOS activated both the MyD88-dependent and -independent pathways through NF-κB and IRF-3 transcription factors, respectively. Moreover, LOS induced the expression of costimulatory molecule CD80 but not CD86 on the surface of monocytes via upregulation of transcription factor IRF-1. These results suggest that phosphoryl moieties of LA from *N. meningitidis* and *N. gonorrhoeae* LOS play an essential role in the determination of the differential activation of both the MyD88- and TRIF-dependent pathways. These findings are consistent with the concept that bacteria modulate pathogen-associated molecular patterns by expression of phosphoryl moieties on the LA to optimize interactions with the host.

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The prognostic significance of detecting endotoxin with Endotoxin scattering photometry (ESP)

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Introduction

Endotoxin scattering photometry (ESP) is a new assay for detecting endotoxin that facilitate to detect very small amounts of endotoxin within 1 hour. This is because ESP identifies directly the clotting enzyme product, coagulin, which is the first appearance of Limulus amebocyte lysate

(LAL) cascade and the precursor of gel clots. Our aim is to verify the correlation between prognostic significance of the endotoxin assay with ESP and SIRS, sepsis or septic shock.

Methods

Nineteen ED patients and forty ICU patients diagnosed with infectious diseases, suspected of having infectious diseases were enrolled in the study, with the addition of 35 healthy volunteers. The whole cohort was divided into groups depending on the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) definitions for SIRS, sepsis and septic shock. All blood samples were examined immediately after mixing LAL reagents, then coagulin particle appearance was measured with a laser scattering platelet aggregometer (model PA-200, Analytical Software, version 3, Kowa, Nagoya, Japan).

Results

The endotoxin prevalence among healthy volunteers was median, 0.010pg/ml 【interquartile range, 0.001-0.383 pg/ml】. On the other hand, the levels of endotoxinwere significantly higher in the patients with septic shock (median, 13.5 pg/ml;interquartile range, 4.67 to 30.9 pg/ml) than in the patient without SIRS(0.209 pg/ml; 0.031 to 0.503 pg/ml). AmongED and ICU patients 69.2 % (n=52) was SIRS, sepsis or septic shock. The area under the receiver operatingcharacteristic curve was 0.917 in the patients with SIRS. Cut-offconcentrations for optimum prediction of SIRS with ESP were endotoxin>0.7 pg/ml.

Conclusions

Our study shows the high diagnostic performance of endotoxin assay with ESP in patients with SIRS. The clinical usefulness of ESP is strengthened by the quickness and the detection of very small amount of endotoxin. It could be tested daily or more as a valid laboratorial help for critical patient's clinical assessment.

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The Bordetella Saga: latest updates on B. pertussis, B. parapertussis, B. bronchiseptica and B. petrii lipids A

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The *Bordetella* genus comprises several human and animal respiratory tract pathogens and nine species are currently described. T he most extensively studied ones are *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*;the recently isolated *B. petrii* is unique, since it is the only species isolated from the environment. Interestingly, it was recently isolated from humans having different infections, including respiratory ones. Despite the availability of vaccines, there are still 400,000 deaths/year caused by whooping cough (*B. pertussis*) and a high morbidity in many countries (*B. pertussis* and *B. parapertussis*). Newly emerged human pathogens (*B. bronchiseptica* and *B. holmesii*) also represent a serious danger. It is also important to note that significant economic losses are associated with infections in poultry and cattle due to *Bordetellae (avium , hinzii*, and *bronchiseptica)* species.

While studying the endotoxin structural diversity and variability, as factors of bacterial adaptation and virulence, we recently established detailed structures of several laboratory strains and clinical isolates of *B. pertussis, B. parapertussis, B. bronchiseptica* and *B. petrii*. We showed that *B. bronchiseptica* and *B. pertussis* were modifying their lipids A by substitution of both phosphate groups with glucosamine residues. Up to now this substitution has not been observed in any other bacterial genus. Its presence was shown to strongly increase the biological activities of purified endotoxin and whole bacteria. The occurrence of palmitate in the lipid A structures of *B. bronchiseptica*, at one or two sites, was correlated with its virulence phase. Other relevant structural features, were the

presence of short fatty acids (C_{10}OH) at C3/C3' positions in different species of the genus.
These demonstrated molecular modifications of *Bordetella* endotoxins may explain how these pathogens adapt to new host ranges and to changes in host dynamics by manipulating the host immunity-pathogen interactions. Moreover, the endotoxin structure can be considered as a good tool to illustrate evolution of this genus.

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Endotoxin structural modifications correlate with virulence in Bordetella bronchiseptica clinical isolates

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The *Bordetella* genus currently contains nine species, most of which are pulmonary pathogens, like *Bordetella pertussis*, the agent of whooping cough. *B. bronchiseptica* is a mammal pulmonary pathogen recently found in humans. *B. pertussis* and a specific lineage of *B. parapertussis* are assumed to have evolved independently from a *B. bronchiseptica*-like ancestor. *Bordetella* lipid A structures have a common bisphosphorylated β-1,6 glucosamine disaccharide backbone with two amide-linked 3-OH C_{14} substituents. The nature and distribution of ester-linked fatty acids have so far proved to be species- or strain-specific and highly variable. *Bordetella* genus shows a remarkable ability to modify lipid A structures by late-steps in their biosynthesis and these modifications can play significant role in modulating host responses to infection. In the present work, we have compared the lipid A structures of two human and one rabbit isolates in their virulent and non-virulent phases. The occurrence of palmitate in these structures at one or two sites was correlated with virulence. The identification of lipid A structures similar to *B. parapertussis* one, in *B. bronchiseptica* isolates, substantiates the evolutionary significance of these isolates, and its ability to phenotypically adapt to various stimuli. The recently identified glucosamine modifications of *Bordetella* lipids A was also found in these isolates. The molecular heterogeneity of *B. bronchiseptica* lipids A may explain how these pathogens adapt to new host ranges and to changes in host dynamics by manipulating the host immunity-pathogen interactions, especially in the context that the strict human pathogen *B. pertussis* is assumed to be derived from a common ancestor of *B. bronchiseptica*

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Characterisation of Bordetella parapertussis PagP

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Previously we demonstrated that *Bordetella bronchiseptica* PagP ($PagP_{BB}$) is a Bvg-regulated lipid A palmitoyl transferase that is required for resistance to antibody-dependent complement-mediated killing in a murine model of infection.
B. parapertussis also contains a *pagP* gene (encoding $PagP_{Pa}$) but its role in lipid A biosynthesis had not been investigated. We show that wild type *B. parapertusis* lipid A comprises a mixture of tri-, tetra-, penta- and hexa-acylated molecules. The penta-acylated molecule contains one and the hexa-acylated molecule contains two palmitates. We show that *B. parapertusis pagP* is required for palmitoylation of the lipid A. Thus, while $PagP_{BB}$ transfers a

single palmitate to the lipid A 3' position, $PagP_{BPa}$ transfers two palmitates to the lipid A 2 and 3' positions. A *B. parapertussis pagP* mutant is more sensitive than WT to killing by antimicrobial peptides. Furthermore, *B. parapertusis pagP* LPS is less potent than WT LPS for stimulating signaling by Tlr4, particularly human Tlr4. We hypothesise that PagP-mediated modification of lipid A regulates the endotoxicity of *B. parapertusis* LPS as a means to modify interactions between the bacteria and its human host during infection.

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A new mild and easy micro-hydrolysis of lipopolysaccharides using triethylamine citrate

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Lipopolysaccharides (LPS) structural characterization usually starts after their extraction from the bacteria and purification. In some cases, due to structural features, long and strong hydrolytic conditions are needed to sever the lipid A / polysaccharide linkage that may result in some degradation in both moieties, such as dephosphorylation, de-O-acylation and loss of other labile substituents. Such modifications lead to artefacts, modification of the native structures, and result in major changes in biological activity. One of the major problems complicating LPS characterization is the aggregation of these amphipathic molecules. Mild hydrolysis conditions such as pH 4.5 in sodium acetate buffer containing 1% SDS was shown to better preserve native LPS structures.
We showed that citric acid was very useful for solubilisation, disaggregation and cation capture. When added to LPS samples, and to matrix preparations, before MALDI-MS analysis, it greatly improved quality of native LPS mass-spectra. At the same time, triethylamine (TEA) is known for its LPS solubilising capacity, that can be used with advantage.
Herein we present a new and efficient mild hydrolysis procedure that is convenient for characterizing lipid A and polysaccharide moieties derived from micro-scale LPS samples using triethylamine citrate. In addition to triethylamine citrate solubilisation capacities, the described methods can be used directly in MALDI mass spectrometry without salt removal. Under the established conditions almost complete hydrolysis (>90 %) was achieved and acid-labile elements like pyrophosphate, ethanolamine phosphate, amino acids and amino sugars are preserved, both in lipid A and polysaccharide moieties. This will be illustrated with LPS isolated from different bacterial species and strains of *Escherichia, Salmonella, Bordetella, Actinobacillus* and *Pseudomonas*.

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Functional and Structural Differences Between Endotoxins of Two Historically Important Bordetella pertussis Strains

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Bordetella endotoxins show remarkable structural variability both among each other and in comparison to other Gram-negative bacteria. Here we demonstrate that in contrast to the common *B. pertussis* laboratory strain and Tohama I derivative BP338, lipooligosaccharide (LOS) from mouse challenge strain 18-323 is a poor inducer of inflammatory cytokines in human and murine macrophages, is greatly impaired in TLR4-mediated activation of NF-κB in transfected HEK-293 cells, and functions as a TLR4 antagonist. Comparison of lipid A structures of *B. pertussis* strains BP338 and 18-323 revealed that 18-323 (i) lacks the ability to modify its lipid A phosphate groups with glucosamine and (ii) is distinct in its acylation at

the C3' position of the lipid A diglucosamine backbone. Our findings have important implications for interpreting previous studies of host defenses to *B. pertussis* infection in mice and *in vitro*.

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Structural and immunobiological studies of lipopolysaccharide from Acetobacter pasteurianus

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Acetic acid bacteria are used for production of fermented food such as vinegar and yogurt. These fermented products are reported to improve human health because of their bacterial components contained. However, the responsible molecules have not been well identified. *Acetobacter pasteurianus* is found in the flora in fermentation of Japanese traditional vinegar, Kurosu. In this study, we separated lipopolysaccharide (LPS) from *A. pasteurianus*, and analyzed its immunobiological activities and chemical structure. *A. pasteurianus* cells were cultured in polypeptone-yeast extract medium. The cells were subjected to phenol-hot water extraction followed by separation using hydrophobic interaction chromatography to obtain LPS fraction. SDS-PAGE profiles of the fraction showed that the LPS was S-type. The fraction stimulated murine TLR4/MD2-mediated NF-kB activation, but the activity was 10^3-fold weaker than that of *Escherichia coli* type LPS. The fraction also weakly activated murine TLR2, probably because of the activity of contaminated lipoproteins. LPS fraction induced TNF-a production in murine PEC. The lipid A part in LPS was prepared by weak acid degradation followed by silica gel column and TLC separation. The chemical structure was analyzed by compositional analysis, NMR, and MS. The lipid A consisted of four sugars, which contained two uronic acids, and penta- or hexa-acyl chains, but no phosphate group. The structure may be responsible for the low endotoxic nature of LPS.

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Biochemical, biophysical and functional characterization of E-coated magnetic nanoparticles (EMNP)

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The interaction of endotoxins (E) with the innate immune system forms the basis of recognition and reaction against many intruding Gram-negative bacteria (GNB) in mammals(1). Potent cell activation by E depends on the activation of the toll-like receptor 4 complex(2,3). E are amphiphilic molecules that in GNB are integral components of the outer leaflet of the outer member and, after extraction, form in aqueous dispersions three-dimensional supramolecular aggregates (i.e., micelles) even at pM-nM concentrations.relevant for biological responses(4).

We have prepared E-coated magnetic nanoparticles (EMNP) as innovative nanoparticle-based tools for biochemical (e.g., effect of E supra-molecular presentation state on the TLR4 activation pathway) and pharmacological applications. We have developed an efficient and reproducible synthetic strategy to prepare E-coated Magnetic Nanoparticles (EMNP) with defined size and shape, making use of HBNPs (Hydrophobic Brush Nanoparticles, magnetic nanoparticles coated with oleic acid moieties) to strongly adsorb E by interacting with the fatty acyl chains of the lipid A moiety. Metabolically labeled E

(i.e., E. coli LPS, meningococcal LOS) in which the fatty acyl chains were uniformly and nearly exclusively radiolabeled were used after sonication to facilitate quantitative monitoring of the amount of E bound/EMNP and the stability of E binding.

The EMNP are stable in solution with no spontaneous release of radiolabelled E from the particles. The EMNP stimulated TLR4-dependent cytokine production (e.g., IL-8, TNF-α) from macrophages, dendritic cells, and transformed HEK293 cells. Cell activation was also CD14-dependent as manifest by reduced induction of cytokine production in CD14 -/- macrophages, and the inhibitory effect of an anti-CD14 compound(5,6).

These findings suggest that the EMNP may provide a new approach for the development of nanoparticle-based vaccine adjuvants or immunostimulators.

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Regulation of Pseudomonas aeruginosa lipid A modifications under oxygen limited growth conditions

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Pseudomonas aeruginosa (PA), an opportunistic pathogen of the airways of patients with cystic fibrosis (CF) has the ability to regulate the structure of the lipid A moiety of lipopolysaccharide (LPS). PA isolated from patients with CF synthesize specific lipid A structures not present in acute and environmental isolates. PA lipid A is composed of a β-(1´-6)-linked glucosamine disaccharide backbone with 12 carbon fatty acids at the 2 and 2' positions and 10 carbon fatty acids at the 4 and 4' positions. Two additional 12-carbon fatty acids are attached at the 2 and 2' positions forming two acyloxyacyl groups, which is carried out by the acyltransferase enzyme, HtrB. Interestingly, the laboratory-adapted strain PAO1 had two genomic copies of the *htrB* gene. To assess the role of each gene, mutants were generated and the lipid A fatty acid content was determined using gas chromatography. Subsequently, complete lipid A structural analysis was determined using higher order mass spectrometry. This analysis clearly showed that the individual *htrB* genes were not redundant with each carrying out a site-specific acylation reaction.

An early adaptation of PA colonization of the CF airway is the addition of palmitate to lipid A. In CF clinical isolates, this modification is

constitutively activated, though inducible in all other isolates upon growth under magnesium limitation requiring the two-component regulatory system, PhoP-PhoQ. The microenvironment in the CF airway has been shown to be oxygen-limited within mucus plugs. We report that under oxygen-limited growth conditions, environmental and acute disease clinical isolates were induced to add palmitate similar to those present in CF clinical isolates. Additionally, we show that palmitoylation requires PhoP-PhoQ, suggesting a novel sensing environment for this regulatory system. Finally, we showed that this is a PA specific process, as other bacterial species did not add palmitate after growth under oxygen-limited conditions.

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Endotoxin Activity Assay (EAA) and Polymyxin-B hemoperfusion (PMX-DHP)- an endotoxin targeted theranostic strategy

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Introduction : Endotoxin (LPS) is a lethal mediator of critical illness. In the EUPHAS trial (JAMA 2009), PMX-DHP was shown to decrease mortality in patients with septic shock. Patients were selected on clinical grounds due to the lack of a tool to reliably measure LPS in vivo. The combination of a clinically useful assay to measure LPS in blood, linked to a specific therapy provides an important theranostic advance for critically ill patients with endotoxemia. We tested (a) the laboratory robustness of the EAA, and (b) its clinical utility to select responders of PMX-DHP. **Methods:** (a) Robustness was defined as resistance to external contamination while providing clinically relevant information. Healthy donor samples (n=8) were assayed before and after exogenous contamination with 230 pg/mL and 1000 pg/mL of E. Coli O55:B5 LPS into the reagents. (b) Clinical utility was tested in an observational study of the EAA (7 centers, Japan, 2007-08. 47 critically patients were selected for PMX-DHP therapy (61 treatments). EAA was drawn at baseline and 12 hours after PMX-DHP). **Results:** (a)Healthy donor baseline EAA 0.24 (mean) and following the addition of 230pg/ml, 0.22 EAA units and 0.26 units following addition of 1000pg/mL. There were no significant differences in EAA values following addition ofexogenous LPS.(b) Mean EAA prior to PMX-DHP was 0.65 units (+/- 0.22) at 12 hours the mean EAA value was 0.45 (+/-0.17) [p<0.010].

Conclusions : The EAA is a robust and rapid (30 min) test for LPS and is resistant to external contamination and is readily applicable for routine clinical practice. This theranostic represents an attractive opportunity to remove endotoxin in patients with proven endotoxemia and may result in improved outcomes.

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Organ specific regulation of inflammatory gene transcription in pigs challenged with wild-type or an LPS-deficient mutant of N. meningitidis

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Background: Fulminant meningococcal sepsis is characterized by overwhelming inflammatory responses to a massive bacterial load (10^6-10^8

meningococci/mL). LPS is the primary, but not the sole inflammation-inducing molecule. While *in vitro* studies of inflammatory responses normally utilize peripheral leukocytes or cell-lines, organs like the liver probably contribute more to the septic inflammation *in vivo.* ***Aim:*** To investigate activation of inflammatory mechanisms by LPS and non-LPS molecules of *N. meningitidis* in whole blood and liver tissue. ***Methods:*** Gene transcription was analyzed by micro array in whole blood and liver from pigs receiving wild-type *N. meningitidis* (NmLPS+) or an LPS-deficient mutant strain (NmLPS-)in a new model of meningococcal sepsis. ***Results:*** Pro- and antiinflammatory cytokines, their respective receptors, chemokines and leukocyte adhesion molecules were in general up-regulated, both in NmLPS+ and in NmLPS- pigs, but significantly more in NmLPS+ pigs. Specifically, TNF-α was equally, but modestly, up-regulated in whole blood and liver. IL-1β and IL-6 were modestly up-regulated in whole blood, but highly up-regulated in liver. IL-10 was up-regulated primarily in whole blood. IL-10R and IL-1RA was up-regulated only in liver. IL-8, MCP-1, MIP-1α, MIP-1β and MIP-2α were primarily up-regulated in liver, whereas the latter three were to some extent up-regulated also in whole blood. E-selectin, VCAM-1 and ICAM-1 were primarily up-regulated in liver, although a modest up-regulation of E-selectin and ICAM-1 also occurred in whole blood. Complement receptor C5aR and complement factor D were down-regulated only in whole blood from NmLPS+ pigs. ***Conclusion:*** The liver contributes substantially more than peripheral leukocytes to the septic inflammatory response. Our data confirm that LPS is the most potent inducer of inflammation in whole blood and the liver, but highlights non-LPS molecules to contribute significantly to the systemic inflammation. Key inflammatory mechanisms, in particular alternative complement activation and complement signaling by C5aR, are down-regulated in sepsis.

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Dynamic multigene families of pattern recognition receptors in the sea urchin suggest a novel form of animal immunity

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In vertebrate and well-characterized invertebrate systems, Toll-like receptors (TLR) and Nod-like receptors (NLR) receptors form small gene families (10-20 genes) that are generally evolutionarily static. In contrast, annotation of the purple sea urchin genome reveals that gene families encoding these receptors are significantly expanded (>200 TLR and NLR genes). Most of the sea urchin TLR genes are structurally similar to vertebrate TLRs and form seven subfamilies based on phylogenetic analysis of the intracellular TIR domains, while ectodomains of the remaining, more divergent, TLRs are either shortened or resemble Drosophila Toll. Levels of variation among subfamily members suggest regions of rapid diversification within the ectodomain that may be associated with ligand interaction or dimerization, and specific residues appear to be under positive selection. The high frequency of pseudogenes and similarity of genes within subfamilies suggest that the many TLR genes result from recent duplication events that is consistent with the model of birth-and-death evolution. TLR subfamilies are differentially expressed in immunocytes, with the highest expression levels observed in the phagocytic subset of immunocytes. Similarly, the complex NLR multigene family is defined by a number of phylogenetic subfamilies that are differentially expressed in gut tissues and immunocytes. The sea urchin NLRs are characterized by slightly different effector domains than their vertebrate counterparts, and most components of the inflammasome pathway are present. Notably, both TLRs and NLRs are expressed in the sea urchin larvae, and are likely important in mediating immunity at this developmental stage. Regulatory mechanisms that evolved to

mediate the complex signaling from these diverse families of immune receptors may have also allowed for the later emergence of vertebrate immunity. This research is supported by the Canadian Institutes for Health Research, the Natural Sciences and Engineering Research Council of Canada, and the Ontario Ministry of Research and Innovation.

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Interleukin-15 is critical in the pathogenesis of eosinophilic esophagitis

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Using global quantitative microarray analysis, we previously reported increased expression of IL-15 mRNA in the esophagus of patients with eosinophilic esophagitis (EE), a recently recognized allergic disorder with poorly understood pathogenesis. Herein, quantitative PCR analysis demonstrates that IL-15 and its receptor IL-15Rα are induced ~6 and ~10-fold in human EE and ~3 and ~4-fold in an experimental murine model of allergen-induced EE, respectively. Interestingly, a >2-fold increase of serum IL-15 protein was also detected in human EE compared to normal individuals. Human IL-15 mRNA correlated ($p<0.001$) with esophageal eosinophilia.Notably, IL-15Rα gene-deficient mice were protected from allergen-induced esophageal eosinophilia compared to the wild type mice ($P<0.001$) even though a comparable airway eosinophilia was observed in the same mice. Further, IL-15 was able to activate STAT5 and promote proliferation and activation of $CD4^+$ T cells to produce eosinophil selective cytokines. In addition, treatment of primary esophageal epithelial cells (from mice and humans) with IL-15 induced a dose dependent increase in the expression of eotaxin-1, eotaxin-2 and eotaxin-3 mRNA. Taken together, we propose a key role for IL-15 in the pathogenesis of EE involving IL-15-induced production of eosinophil active cytokines by $CD4^+$ T cells and chemokines by esophageal epithelial cells.

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A Chemical Genetic Screen to Identify Modulators of the Neutrophil Response During Inflammation

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An effective inflammatory response is dependent upon trafficking of innate immune cells to affected tissues. These migratory cells include early-arriving neutrophils, short-lived leukocytes that provide a potent yet non-specific defence against invading pathogens and more long-lived monocytes/macrophages, that arrive slightly later and remove cellular debris including 'spent' neutrophils. Signals that manage the migration of neutrophils to inflamed tissue and their persistence within these sites need to be precisely regulated. Further understanding these signals is central to enhancing the therapeutic control of neutrophil-driven inflammatory conditions, such as rheumatoid arthritis and osteoarthritis. The immune system of zebrafish demonstrates remarkable conservation with that of mammals, possessing a full complement of innate and adaptive immune cell lineages. Furthermore, the later onset of a functional adaptive immune system during zebrafish development enables the contribution of innate immune cells to be exclusively examined in response to inflammation. These attributes, combined with live cell imaging of fluorescent immune cell subsets within intact transparent transgenic embryos and genetic tractability, has helped establish the zebrafish as an extremely valuable system in which to investigate immune cell function. More recently, the zebrafish has been exploited, with much success, as a platform to

screen chemical libraries to identify candidate drugs with specific biological activities. We have developed a 'high content' chemical screen to identify small molecules that influence leukocyte recruitment during inflammation. Wounds within transgenic zebrafish larvae, possessing fluorescent neutrophils, results in a quantifiable and reproducible neutrophil infiltration of wounded tissues. We are screening several small molecule libraries that cover a broad spectrum of active compounds with high chemical and pharmacological diversity, to assess their ability to alter neutrophil infiltration of wounds. To date, this screen has revealed a remarkable conservation of activity of known anti-inflammatory drugs validating the approach and its potential to uncover novel drugs with immunomodulatory activities.

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The human autoantigen Hom s 2 has potent immunomodulatory effects

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Hom s 2, the alpha-chain of the nascent polypeptide-associated complex, is an intracellular autoantigen that has been shown to exhibit both IgE and T-cell mediated immune responses in patients suffering from atopic dermatitis. Recently, Hom s 2 has been shown to induce the release of IFN-gamma in cultured peripheral blood mononuclear cells (PBMC) suggesting that in addition to its role as an autoantigen, it also has intrinsic immunomodulatory capacity. In order to further investigate its immunomodulatory functionswe have studied in detail the cytokine profile (detected by sandwich ELISA) after stimulation of isolated human PBMC, monocytes and CD4+ T-cells with Hom s 2. We detected elevated levels of IFN-gamma and TNF-alpha in culture supernatants of human PBMC stimulated with Hom s 2. There was no difference in the levels of IL-2, IL-4 and IL-5 and IL-13 . Blocking antibodies against IL-12 abrogated the IFN-gamma secretion. Isolated CD 4+ T cells failed to secrete IFN-gamma after stimulation with α-NAC suggesting a role for monocytes. Monocytes stimulated with Hom s 2 readily induced the secretion of IL-12p40. IL-2 enhanced Hom s 2 induced IL-12p40 secretion in monocytes and IFN-gamma secretion in PBMC. Hom s 2 induced IL-12p40 was dependent on the phosphorylation of STAT 3. Monocytes stimulated with Hom s 2 enhanced the expression of cell surface antigens such as CD14, CD40, CD54 and CD80. We could also show that keratinocytes after stimulation with IFN-gamma released Hom s 2 into the culture supernatant. In conclusion, Hom s 2 appears to have potent immunomodulatory functions and may serve as an early 'alarmin' in the immuneresponse of the skin.

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Late Breaking Abstracts

LB1

Detection of Intracellular Bacterial Pathogens by Inflammasome NLRs

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One area of research my laboratory focuses on is the study of how the innate immune system recognizes intracellular bacterial pathogens and how these pathogens manipulate the host immune response. We use genetic and biochemical approaches to identify bacterial and host molecules involved in these complex interactions. By comparing and contrasting the innate immune response to two Gram-negative facultative intracellular pathogens, *Salmonella* and *Francisella*, we have learned a great deal about how the intracellular complex, called the inflammasome, recognizes foreign invaders. Both of these pathogens activate the inflammasome, which is comprised of an effector enzyme (Caspase-1), an adaptor protein (ASC) and an intracellular NOD-like receptor (NLR). The activated inflammasome cleaves pro-inflammatory cytokines and sometimes leads to macrophage death. Although both *Salmonella* and *Francisella* activate the inflammasome, the molecular mechanisms are different, which could reflect their different intracellular location and/or different pathogenic strategies. For example, we have recently shown that the NLRs NLRP3 and NLRC4 both activate caspase-1 in response to intracellular *Salmonella*, which reside in a specialized vacuole in macrophages. Indeed, mice lacking both NLRs are markedly more susceptible to infection, revealing an unexpected redundancy among NLRs in host defense against intracellular pathogens during infection. In contrast, NLRP3 and NLRC4 are not involved in recognizing intracellular *Francisella*, which reside in the cytosol of macrophages. We have recently shown that intracellular *Francisella* are recognized by the NLR AIM2. We have shown that AIM2 binds bacterial DNA in the cytosol and recruits ASC and caspase-1 into a single cytoplasmic focus, which serves as the site of pro-IL-1 β processing. Finally, we show that mice deficient for AIM2 are more susceptible to *Francisella* infection compared to wild-type mice. Ultimately, a better understanding of the molecular mechanisms of inflammasome activation and how bacterial pathogens modulate these pathways may lead to a rational design of new therapies that target specific innate immune pathways.

LB2

Interaction of Lipopolysaccharide-binding protein (LBP) with human host cell membranes

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The Lipopolysaccharide-binding protein (LBP) is an acute-phase protein mediating the immune response to Gram-negative lipopolysaccharide (LPS). Currently, LBP is mainly known as a serum protein which transfers LPS to sCD14, subsequently activating the TLR4/MD-2 receptor complex. This triggers an immune response potentially leading to sepsis, one of the major causes of death in intensive care units. Recently, our group showed that LBP intercalates into phospholipid bilayers. Our current work implicates that LBP also interacts with host cell membranes. However, the properties of the LBP interaction with eukaryotic cell membranes are still unknown. We strive to investigate the mechanisms underlying this interaction. In *Förster* resonance energy transfer (FRET) experiments on reconstituted lipid membranes we could show that LBP integrates into phospholipid liposomes depending on lipid surface charge and ion concentration. Flow cytometry analysis of human macrophages demonstrated that LBP not only binds to the macrophage cell membrane but subsequently is quickly taken up into the cells. These results

were confirmed with immunofluorescence stainings of HEK293-hTLR4/MD2 cells which also bound and internalized LBP. In fractioned cell extracts of human macrophages, LBP could be found in membrane fractions and was enriched in lipid raft fractions following LPS stimulation of the cells. Our results suggest that besides its shuttle function LBP directly interacts with the host cell. Therefore, we propose that LBP is not exclusively a serum protein but also binds and possibly integrates into the plasma membrane of host cells. Our results, showing that LBP accumulates in cholesterol rich domains, lead us to the assumption that the protein might play a role in the "lipid raft" signaling concept. Acknowledgements: This work is funded by the Leibniz Graduate School "Model Systems for Infectious Disease".

LB3

A new approach in Sepsis treatment based on synthetic anti-LPS peptides (SALP)

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The increasing number of antibiotic resistant bacterial strains is a severe problem in treating SEPSIS. Therefore, it is necessary to find new defense strategies and treatment options. In this context the work is dedicated to the analysis of several newly synthesized anti-LPS peptides (SALPs). These peptides are synthetic compounds designed to effectively neutralize lipopolysaccharide (LPS), and hence, act as new potential drugs. Preliminary biophysical studies using isothermal titration calorimetry (ITC) showed that all SALPs are able to bind LPS at low concentrations with high affinity. Following in vitro studies on LPS-stimulated macrophages clearly showed an inhibition of TNFalpha and Il-6, which are cytokines known as key players during the immune reaction causing sepsis. Furthermore, we could support these results in in vivo studies. Mice with LPS-induced sepsis symptoms could be cured with subsequent administration of SALPs. Additionally, a cecal ligation and puncture (CLP) model confirmed our data. To ensure the medical applicability, cytotoxicity analyses have been performed using LDH and MTT as well as hemolysis and Alamar blue® assays. All investigated peptides showed no cytotoxicity at concentrations, which completely neutralized LPS. Therefore, we propose that these peptides lead to the neutralization of the endotoxin and can be used in clinical trials for the therapy of sepsis patients.

LB4

Gastric Inflammation and Tumorigenesis are Promoted by Toll-like Receptor 2 Signaling in the gp130Y757F/Y757F Mouse Model

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Gastric cancer (GC) is the second most lethal cancer world-wide, and while a strong link has been established between colonization of *Helicobacter pylori* and chronic gastritis and GC, a significant number (20-25%) of human GC cases develop in the absence of *H. pylori*, albeit be poorly understood mechanisms. Deregulated activation of cytokine signaling pathways, especially the latent transcription factor signal transducer and activator of transcription (STAT)3, is implicated in various inflammation-associated cancers, including up to 50% of human GC cases. However, the molecular consequences of aberrant STAT3 activation in promoting inflammation and carcinogenesis remain to be fully elucidated. To provide novel insights into such pathological mechanisms in vivo, we have employed a genetic approach based on a mouse strain ($gp130^{Y757F/Y757F}$) carrying a specific "knock-in" mutation in the IL-6/IL-11 cytokine family co-receptor gp130 which prevents negative regulation of gp130-dependant signaling. These mice develop spontaneous gastric inflammation

and gastric tumors in an IL-11- and STAT3-dependant manner, which histologically resemble intestinal-type metaplastic human GC. We report a novel role for Toll-like receptor (TLR)2,a receptor responsible for recognising diverse bacterial ligands and triggering inflammatory responses, in gastric tumorigenesis in $gp130^{Y757F/Y757F}$ mice. Expression of TLR2 was found to upregulated in the stomachs of $gp130^{Y757F/Y757F}$ mice and genetic ablation of TLR2, but not other TLRs, in $gp130^{Y757F/Y757F}$ mice alleviated tumor formation. The role of TLR2 was further analysed in mice deficient in the TLR2 and TLR4 singaling adaptor protein, MyD88-adaptor like (Mal). Interestingly, tumorigenesis in $gp130^{Y757F/Y757F}$:$Mal^{-/-}$ mice was equivalent to their $gp130^{Y757F/Y757F}$ littermates, echoing recent data indicating that Mal is not essential for TLR2 signaling. Finally, we also show that TLR2 expression was found to be increased in human gastritis and GC biopsies. Collectively, our data indicate a key role for the innate immune system in the development of inflammation associated GC.

This work is supported by the National Health and Medical Research Council of Australia, Cancer Council of Victoria, Monash University and Association for International Cancer Research.

LB5

The effect of resolvin D1 on D-galactosamine-primed endotoxin shock model in mice

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Endotoxin shock is a severe systemic inflammatory response that is caused by the augmented production and release of septic mediators. Among them, inflammatory cytokines and chemokines such as TNF-α, IL-6, MCP-1 play a pivotal role. In addition, high mobility group box-1 (HMGB1), a non-histone chromosomal protein has recently been recognized as member of septic mediators. Resolvins are a family of potent anti-inflammatory lipid mediators derived from both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Resolvin D1 (RvD1) is produced physiologically from the sequential oxygenation of DHA by 15- and 5-lipoxygenase. RvD1 reduces human neutrophils transendothelial migration, and reduces neutrophil infiltration in a murine peritonitis model. However, it is unclear whether RvD1 exhibits protective action on endotoxic shock. In this study, to elucidate the effect of RvD1 on endotoxin shock, we investigated the action of RvD1 on septic mediators, numbers of peritoneal exudate cells, and hepatocyte apoptosis using D-galactosamine-primed endotoxin shock model. Administration of LPS (25 ng/mouse) and D-galactosamine (18 mg/mouse) enhanced the serum levels of septic mediators (TNF-α, IL-6, MCP-1, IL-10 and HMGB1). Of note, RvD1 (1 μg/mouse) suppressed the LPS-induced increase of MCP-1 and HMGB1. RvD1 also suppressed the LPS-induced accumulation of neutrophils and reduction of mononuclear cells in the peritoneal cavity. Moreover, RvD1 moderately inhibited the apoptosis of hepatocyte. Together these observations indicate that RvD1 may exhibit protective action on endotoxin shock via suppressing inflammatory mediators, neutrophil accumulation, and hepatocyte apoptosis.

LB6

Roles of IL-17A/F in Inflammatory Immune Responses and Host Defense against Pathogens

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IL-17A is a cytokine produced by Th17 cells and plays important roles in the development of allergic and autoimmune diseases. Although IL-17F is highly homologous to IL-17A and binds the same receptor, the functional roles of this molecule remain largely unknown. We found that IL-17A, but not IL-17F, plays major roles in

the development of delayed-type and contact hypersensitivities, experimental autoimmune encephalomyelitis, OVA-induced pulmonary inflammation, collagen-induced arthritis, and arthritis that spontaneously develops in *Il1rn*$^{-/-}$ mice. Interestingly, *Il17a/f*$^{-/-}$ mice showed increased susceptibility to opportunistic infection by *Staphylococcus aureus*. Furthermore, both *Il17a*$^{-/-}$ and *Il17f*$^{-/-}$ mice showed increased susceptibility to *Citrobacter rodentium* infection, suggesting that both IL-17A and IL-17F play important roles in the host defense against mucoepithelial infection. We also found that IL-17A, but not IL-17F, plays an important role for the host defense against systemic *Candida albicans* infection. We found that the β -glucans on the fungus were recognized by Dectin-1, while α -mannans were recognized by Dectin-2, by using knockout mice of these genes. Although the susceptibility of *Dectin-1*$^{-/-}$ mice to *C. albicans* were similar to wild type mice, *Dectin-2*$^{-/-}$ mice were highly susceptible to systemic candida infection, suggesting that Dectin-2, but not Dectin-1, is important for the host defense against this fungus. Both yeasts and hyphae induced Th17 differentiation, in which Dectin-2, but not Dectin-1, was mainly involved. These observations indicate that IL-17A and IL-17F have overlapping yet distinct roles in host immune and defense mechanisms.

LB7

Phosphatidic Acid (PA) is a leukocyte chemoattractant

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Phosphatidic acid (PA) is a pleiotropic lipidic second messenger in mammalian cells. We report here that extracellular PA acts as a leukocyte chemoattractant, as membrane-soluble dioleoyl-PA (DOPA) elicits actin polymerization and chemotaxis of human neutrophils and differentiated proleukemic HL-60 cells. We show that the mechanism for this involves the S6 kinase (S6K) signaling enzyme. Chemotaxis was inhibited >90% by the S6K inhibitors rapamycin and bisindolylmaleimide, and by S6K1 silencing using dsRNA. However, it was only moderately (~30%) inhibited by mTOR siRNA, indicating the presence of an mTOR-independent mechanism for S6K. Exogenous PA led to robust time- and dose-dependent increases in S6K enzymatic activity and T421/S424 phosphorylation, further supporting a PA/S6K connection. We also investigated whether intracellular PA production affects cell migration. Overexpression of PLD2 and, to a lesser extent, PLD1, resulted in elevation of both S6K activity and chemokinesis, whereas PLD silencing was inhibitory. Since the lipase-inactive PLD2 mutants K444R and K758R neither activated S6K nor induced chemotaxis, intracellular PA is needed for this form of cell migration. Lastly, we demonstrated a connection between extracellular and intracellular PA. Using an EGFP-derived PA sensor, we showed that exogenous PA, or PA generated in situ by bacterial (Streptomyces chromofuscus) PLD enters the cell and accumulates in vesicle-like cytoplasmic structures. In summary, we report the discovery of PA as a leukocyte chemoattractant, via cell entry and activation of S6K to mediate the cytoskeletal actin polymerization and leukocyte chemotaxis required for the immune function of these cells.

LB8

Human Circulating Eosinophils Constitutively Store, and Rapidly and Differentially Secrete, Th1, Th2 and Immunoregulatory Cytokines

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Eosinophils are innate immune leukocytes with newly recognized roles in immunomodulation and tissue remodeling. Many of these functions derive from the capacity of eosinophils to rapidly secrete varied cytokines, stored preformed within intracellular granules. Preformed cytokines are secreted from intracellular granules through vesicle-mediated transport. We previously visualized the emergence of vesicles from intracellular granules using 3-dimensional electron tomography, and identified a mechanism by which IL-4Rα chains chaperone bound IL-4 into

secretory vesicles for release. Despite a favored association of eosinophils with Th2 immunity, eosinophils from atopic donors can express over 30 cytokines, including Th1-associated cytokines. However, whether or not all of these cytokines are constitutively stored preformed within granules, and therefore readily available for immediate release from eosinophils within circulation, had not been determined. In the current study, we used multiplex analyses to investigate the cytokine potential of circulating eosinophils, isolated from normal donors and those with histories of mild atopy. Our results demonstrate that human eosinophils from atopic and non-atopic donors constitutively store a cache of cytokines with nominal Th1, Th2 and regulatory effects, including IL-4, IFN-γ, IL-12, IL-10 and TNF- α. Relative concentrations of individual cytokines stored within human eosinophil granules were highly reproducible amongst donors (with the notable exception of TNF-α). In response to a variety of immune-polarizing conditions, rapid and differential secretion of cytokines was observed. Notably, Th1 and inflammatory agonists elicited release of Th2-promoting IL-4, but not Th1-inducing IL-12, and large quantities of IFN-γ were readily released from eosinophils in response to a variety of stimuli. Determinations of the multifarious nature of the cytokine potential of eosinophils within circulation, and delineations of the profiles of rapid cytokine secretion under the pressure of different immunomodulating cytokine environments provide key insights into the functions of human eosinophils in mediating inflammation and modulating immunity.

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LB9

Common and unique structure and functions of Toll-like receptors in the ascidian *Ciona intestinalis*: insights into molecular and functional evolution of deuterostome Toll-like receptors

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Toll-like receptors (TLRs) play a pivotal role in host defense responses to invading microbial pathogen. Recent genomic surveys have detected a great variety of TLR-like genes in deuterostome invertebrates. However, their functions, localization and ligand recognition, and signalling remained to be elucidated. The ascidian, *Ciona intestinalis*, is the invertebrate chordate most phylogenetically close to vertebrates and regarded as the best model organisms for studies of the evolution of innate immunity. In this study, we present the structures, cellular localization, ligand specific activities, and biological functions of two *Ciona intestinalis* TLRs, Ci-TLR1 and -2. We cloned Ci-TLR1 and Ci-TLR2 from *Ciona* hemocytes. SMART protein domain analysis revealed that each of the deduced protein has a TIR domain, a single transmembrane domain and multiple leucine rich repeats, which are reminiscent of mammalian TLRs. Ci-TLR1 and Ci-TLR2 expressed in the HEK293 cells, unlike mammalian TLRs, were located to both the plasma membranes and endosomes. Futhermore, Ci-TLR1 and Ci-TLR2 activated NF-κB in response to multiple pathogenic ligands such as dsRNA, flagellin and bacterial cell wall components. *In situ* hybridization and RT-PCR demonstrated that Ci-TLR1 and Ci-TLR2 genes were expressed predominantly in the stomach, intestine and hemocytes. The aforementioned Ci-TLR ligands also induced an increase of Ci-TNFα production in the stomach, intestine and hemocytes where Ci-TLRs are expressed. These results provide that the TLR-triggered innate immune systems are conserved in chordates, and that Ci-TLRs are functionally "hybrid" TLRs of

mammalian cell-surface TLRs and endosome TLRs. Based on these finding, we also present the possible evolutionary processes of TLRs and related genes.

LB10

Microglial phenotype modulation after TBI via progenitor cell:splenocyte interactions

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We have shown that intravenously transplanted progenitor cells and resident splenocyte interactions can modulate the immunological response to traumatic brain injury (TBI) in a rodent model. MAPCs (multipotent adult progenitor cells) localize to the spleen, and preserve splenic mass by inhibiting splenocyte apoptosis and increase proliferation. These splenocytes increase anti-inflammatory cytokine output (IL-4 and 10) and preserve blood brain barrier integrity/tight junctions. This study sought to determine if MAPCs increased Tregs in the spleen, and if this correlated with changes in the brain microglia/macrophage phenotype from the M1/"inflammatory-classical" activated state to the M2/"reparative-alternate" activated state. Methods: C57 Black 6 mice underwent controlled cortical impact TBI or sham and one group of injured animals had 10 million MAPC/kg body wt. injected at 2 and 24 hours after injury. Blood brain barrier integrity was measured at 72 hours after injury. Treg appearance in the spleen and circulating blood were measured. CD 86+ (M1) and CD 206+ (M2) brain microglia/macrophage populations were quantified in the brain at 24, 48, 72, and 120 hours after TBI. Results: BBB permeability was reversed with MAPCs and this correlated with and increase in splenocyte and circulating Tregs relative to controls. There was a significant increase in the M2/M1 ratio of microglia/macrophage in the MAPC treatment group at 48 and 120 hours after injury. Conclusions: MAPCs interact with splenocytes to increase the anti-inflammatory Treg population. This correlates with a down-regulation of the typical M1 phenotype in the brain after injury and preserves the integrity of the blood brain barrier. This novel mechanism of action has implications regarding the timing, delivery route and effect of progenitor cells administered after injury.

LB11

Aryl hydrocarbon receptor regulates Lipopolysaccharide-induced Inflammation in Macrophages through suppressing Histamine production

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Histamine, which is produced by histidine decarboxylase (HDC), is known to be involved in inflammation, allergic reactions, gastric acid secretion, and neurotransmission via Histamine 1-4 receptors (H1-4R).Histamine regulation is dependent on HDC activity in various cells. In mast cells and basophils, it is well known that the HDC transcriptional activity is regulated by CpG metylation in the promoter region. However, the mechanism of lipopolysaccharide (LPS)-induced HDC expression in macrophages is not well known. In this study, we found that Aryl hydrocarbon receptor (Ahr), which was induced by LPS, inhibits the expression of HDC in macrophages. Ahr is not only well known as a mediator of chemical toxicity, but also involved in immune system. We investigated how Ahr regulates LPS-induced HDC induction, using peritoneal macrophages or RAW 264.7 overexpressing Ahr (RAW/Ahr) and RAW/Neo as control. As it is reported that Sp1 induces the expression of HDC in activated macrophages by LPS, we concretely examined whether Ahr is involved in Sp1 activation. Then, we found that Ahr attenuated the binding activity of Sp1 to the

GC box region in the mouse HDC gene promoter through forming a complex with Sp1. As a result of the inhibition of Sp1 activation, we found that the production of histamine was significantly suppressed in RAW/Ahr, compared to that in RAW/Neo and elevated in Ahr deficient ($Ahr^{-/-}$) macrophages. Next, we examined the effects of histamine on the secretion of inflammatory cytokines of macrophages. Then, we found histamine H1 receptor antagonists inhibit LPS-induced IL-6 production of macrophages. Thus, Ahr may also negatively regulate LPS-induced pro-inflammatory cytokines through inhibiting histamine production of macrophages as we previously demonstrated that Ahr in combination with Stat1 regulates LPS-induced inflammatory cytokines.

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LB12

In vivo stimulation of γ9+ T-cells by phosphoantigen increases their response to acute bacterial infection in the marmoset (Callithrix jacchus)

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$\gamma9\delta2^+$ T-cells are exclusive to primates species and are involved in killing intracellular bacteria. $\gamma9\delta2^+$ T-cells respond to bacterial phosphylated metabolites known as phosphoantigens that have been developed as potential immunotherapies for infectious diseases. *Burkholderia pseudomallei* is an intracellular bacterial pathogen, which causes the disease melioidosis. Melioidosis is a major public health problem and a biodefense threat agent. Interferon-γ (IFN-γ) is essential for host defence against *B. pseudomallei*. We aimed to evaluate whether stimulation of $\gamma9\delta2^+$ T-cells using the synthetic phosphoantigen IPH1201, co-administered with interleukin-2, can protect against lethal *B. pseudomallei* infection in the common marmoset (*Callithrix jacchus*). We initially demonstrated that subcutaneous injection of IPH1201 caused a 10-fold increase in total peripheral and splenic $\gamma9^+$ T-cell numbers (both IFN-γ^+ and IFN-γ^-), which peaked 5 days following treatment. At this time-point, marmosets were challenged by aerosol exposure with a 100 lethal doses of *B. pseudomallei* strain K96243. Both peripheral and systemic $\gamma9^+$ T-cell responses were identified following infection with an increase in $\gamma9^+$ T-cells in the blood, lung, liver and spleen. *B. pseudomallei* caused an increase in the proportion of both $\gamma9^+$ and $\gamma9^-$ T-cells expressing IFN-γ. This suggests a possible 'bystander effect' of the treatment on other $\gamma9^-$ T-cell populations. Despite these promising results, we found that this treatment did not result in protection; even though the therapy appeared to limit bacterial persistence in the lungs. In conclusion, while the phosphoantigen IPH1201 had a significant impact on relevant immunological parameters, it was unable to induce protective responses in this acute infection model.

LB13

Aryl hydrocarbon receptor mediates suppression of colon inflammation induced dextran sulfate sodium

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Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract. It has become clear that Th1 or Th2, cause worsening symptoms, and moreover that IBDs are

characterized by a sustained production of cytokines produced by newly identified Th17 cells. Our group and others have demonstrated that Aryl hydrocarbon receptor (Ahr) participates in Th17 cell differentiation and modulates the Th1/Th2 balance. In this study, we investigated the role of Ahr in the development of dextran sulfate sodium (DSS)-induced colitis. Wild type (WT) and Ahr deficient (KO) mice were given 1% DSS dissolved in drinking water. All Ahr KO mice, but not WT littermates, died within 9 days after DSS administration. Moreover, significant body weight loss in Ahr KO mice was observed following 4 days of DSS administration. Conversely, WT mice receiving 1% DSS showed no loss in body weight. Flow cytometry was used to determine the differentiation of T cells in colonic lamina propria lymphocytes (LPLs) from 1% DSS-treated WT and Ahr KO mice for 5 days. A significant increase in the percentage of Th1 cells was observed in DSS-treated Ahr KO mice compared with DSS-treated WT mice. However, there was no difference in Treg and Th17 cell development in LPLs from DSS-treated WT and Ahr KO mice. Furthermore, we found that pro-inflammatory cytokines including IL-12 were increased, in colon-cultured supernatant of 1%DSS treated Ahr KO mice compared to that of WT mice. It has also been reported that Ahr regulates the production of LPS-induced pro-inflammatory cytokines including IL-12, which induces Th1 cell development. Ahr may inhibit the development of Th1 cells through regulating IL-12 secretion from activated macrophages, which may result in DSS-induced colonic inflammation. We intend to further investigate the mechanistic detail of Ahr function in DSS-induced colitis using macrophage- and T cell-specific Ahr deficient mice.

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LB14

Aryl hydrocarbon receptor deficiency significantly suppresses collagen induced arthritis in mice

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Aryl hydrocarbon receptor (Ahr), as known a dioxin receptor, is a ligand-dependent transcription factor. It has been reported that Ahr plays an important role in the immune system. Our recent study demonstrated that Ahr participates in Th17 cell development and regulates inflammatory responses by lipopolysaccharide (LPS) in macrophage. In this study, we investigated the effect of Ahr on collagen-induced arthritis (CIA). We found that CIA was significantly suppressed in Ahr-deficient (KO) mice, compared to that in wild-type (WT) mice. Although WT mice with CIA showed the marked cartilage destruction, that histopathologic features were milder in Ahr KO mice than in WT mice. To determine which types of immune cells are important for the inhibition of the CIA development by Ahr-deficiency, we employed the conditional knockout strategy. Ahr gene was specifically deleted in macrophages or T cells by crossing Ahr-flox/flox mice with LysM-cre mice or Lck-cre mice. T cell specific Ahr deficient (Lck-cre $Ahr^{flox/flox}$) mice were significantly protected from CIA development. Moreover, the frequency of Th17 cells was decreased in Lck-cre $Ahr^{flox/flox}$ with CIA. In macrophage specific Ahr deficient (LysM-cre $Ahr^{flox/flox}$) mice, CIA development was partially blocked and serum IL-6 level was significantly reduced, compared with those in WT mice . These results suggest that Ahr deficiency in T cells is more important for the suppression of CIA development than its deficiency in macrophage and rheumatoid arthritis may be a T cell dependent disease.

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Late Breaking Abstracts

LB15

Respiratory Syncytial Virus infection is associated with a heightened pro-inflammatory response in the lungs of pre-term lambs

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Factors explaining the greater susceptibility of pre-term babies to severe lower respiratory infections with respiratory syncytial virus (RSV) remain poorly understood. Fetal/newborn lambs are increasingly appreciated as a model to study key elements of RSV infection in newborn infants due to similarities in lung alveolar development, immune response, and susceptibility to the virus. Previously, our laboratory demonstrated that the ability to clear bovine RSV (bRSV) antigen from the lung was age-dependent. Here, we investigated the relationship between developmental age and immunological competence in response to RSV infection in the lung. Lambs were delivered pre-term by Caesarian section at 138 days of gestation or full-term (147 days gestation) by natural birth. Both groups were inoculated with bRSV via the intratracheal route. Seven days post-infection, the right middle lung lobes were collected for viral load, histopathology, cellular infiltration and cytokine production. bRSV antigen, was higher in pre-term than full-term animals. Following infection, increased IL-8, MCP-1, MIP-1α, IFN-γ and PD-L1 were observed in pre-term and full-term animals. This increase was more pronounced in the pre-term than the full-term group. Pre-term lambs infected with bRSV had similar numbers of macrophages as full-term lambs, but reduced nitric oxide levels indicating decreased macrophage function. While caspase 3 immunoreactivity (as a measure of apoptosis) occurred in bronchioles and alveoli of all infected lambs, immunoreactivity was increased in lungs of pre-term lambs. Correlation analysis of the qPCR data showed that the expression of all targets assessed did not differ in control animals regardless of age, suggesting that in an environment devoid of viral stimulation, the lung milieu of pre-term animals is not different from that of full-term animals. Our data indicate that pre-term lambs exposed to bRSV have increased viral load and a heightened pro-inflammatory response, which possibly serve as contributing factors to RSV immunopathology.

LB16

Regulation of Neutrophil Senescence by MicroRNAs

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Neutrophils are rapidly recruited to sites of tissue injury or infection, where they protect against invading pathogens. Neutrophil functions are limited by a process of neutrophil senescence, which renders them unable to respond to chemoattractants, carry out respiratory burst, or degranulate. In parallel, aged neutrophils also undergo spontaneous apoptosis, followed by their subsequent removal by phagocytic cells such as macrophages, thereby preventing unwanted inflammation and tissue damage. Regulation of senescence and lifespan is critical to regulation of neutrophil function and hence inflammation. Key neutrophil anti-apoptotic proteins (e.g. Mcl-1) have been shown to be regulated by miRNAs, a family of short RNA species that negatively regulate gene expression. We therefore hypothesised that neutrophil functional lifespan might be regulated by microRNAs within human neutrophils. Total RNA from highly purified neutrophils was prepared and subjected to microarray analysis using the Agilent human miRNA microarray V3. We found that human neutrophils express a selected repertoire of 148 microRNAs and that 6 of these were

significantly upregulated after a period of 4 hours in culture. A list of predicted targets for these 6 microRNAs was generated from http://mirecords.biolead.org/ and compared to those mRNA species downregulated over time, revealing 83 genes targeted by at least 2 out of the 6 regulated microRNAs. Pathway analysis of genes containing binding sites for these miRNAs identified the following pathways (EASE score $p<0.05$): chemokine and cytokine signalling, Ras pathway, and regulation of the actin cytoskeleton. Our data therefore suggest that microRNAs may play a role in the regulation of neutrophil senescence and further suggest that manipulation of miRNAs might represent an area of future therapeutic interest for the treatment of inflammatory disease. This work was funded by an MRC Senior Clinical Fellowship to SAR (reference number: G0701932).

LB17

Sequential Stimulation of TLR-2 and TLR-4 leads to strong TH1-polarisation and high CCR7 Expression in human monocyte-derived Dendritic Cells

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Dendritic cells (DCs) provide key regulatory signals to T-lymphocytes for an adequate anti-tumour response. DCs may branch into two functional directions designated as T_H1- and T_H2-polarisation, characterised by production of either IL-12p70 or IL-10, respectively. IL-10 is acting as a crucial factor for immune suppressive Type 1 regulatory T cells (Tr1) and as an inhibitor of an efficient anti-tumour immune response. In contrast IL-12p70 is key cytokine for a T_H1- mediated immune response which promotes the activity of tumour-specific cytotoxic T-lymphocytes. Here we described a method to improve the maturation and T_H1-polarisation of MoDCs by sequential stimulation of different Toll like receptors (TLRs). Human DCs generated from peripheral blood monocytes by stimulation with GM-CSF and IL-4 (MoDCs) are commonly used as a source of DCs for immunotherapies against cancer. For that purpose it is important to manipulate efficiently MoDCs to express and up-regulate relevant surface markers and to secrete cytokines favouring a T_H1-cytokine environment. Stimulation of immature MoDC with non-toxic concentration of lipopeptide (Lp) via TLR-2 followed by highly purified LPS preparation GA1-6 or Monophoshoryl-Lipid A (MPLA) via TLR-4 in combination with IFN-γ induced DC maturation accompanied by the production of high levels of IL-12p70 and low levels of IL-10. Interestingly, MoDCs sequentially matured by TLR2 and TLR4 agonists in combination with IFN-γ not only show high expression of co-stimulatory molecules (e.g. CD80 and CD86) and of the maturation marker CD83 but also a high expression of CCR7 despite the absence of PGE_2 or LTB4, respectively. Functionally, these MoDCs exhibited higher antigen-specific T-cell stimulatory activity than MoDCs matured by Poly (I:C) or TNFα/IL-1ß/IL-6/PGE_2.

LB18

Aryl Hydrocarbon Receptor Regulates Immunogenic DC by a Kynurenine Dependent Mechanism

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Aryl hydrocarbon receptor (A hr) plays an important role in immune responses of T cells and macrophages. However, its function in dendritic cells (DC) remains unclear.

Indoleamine 2,3-dioxygenase (IDO), an enzyme converting tryptophan (Trp) into kynurenine (Kyn) and other metabolites, is induced in DC by immune activation. IDO^+ DC are known as tolerogenic DC that involve in Treg cell development. There is accumulating evidence that several tryptophan metabolites can be potent Ahr agonists. Therefore, we hypothesize that there is a potential link between Ahr and IDO in DC that drives naïve T cell differentiation and proliferation. We investigated Ahr and IDO expression in bone marrow-derived dendritic cells (BMDC) from wild-type (WT) and Ahr-deficient ($Ahr^{-/-}$) mice under LPS and CpG stimulation. We found that LPS and CpG stimulate both Ahr and IDO expression in WT BMDC. But IDOmRNA was not induced in $Ahr^{-/-}$ BMDC by LPS and CpG stimulation. Consequently, Kyn level was significantly reduced in $Ahr^{-/-}$ compared to WT BMDC. Interestingly, IL-10 production was abrogated in $Ahr^{-/-}$ compared to WT BMDC. Importantly, we demonstrated that LPS- and CpG-stimulated $Ahr^{-/-}$ BMDC inhibit regulatory T (Treg) cell and promote Th17 cell generation from naïve T cells. Addition of exogenous Kyn skews naïve T cells differentiation towards Treg and away from Th17 cells. Summarily, our results demonstrate a novel function of Ahr in regulating immunogenic function of DC under LPS and CpG stimulation to drive naïve T cell proliferation via a Kyn-dependent mechanism. This work was supported by Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and Chugai-Roche Pharmaceutical Co. Ltd, Tokyo, Japan

LB19

Network Analysis of Gene Expression Response to LPS and Synthetic Immunomodulatory Peptides

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Synthetic innate defense regulator peptides are potential new therapeutic agents for treatment of infections that act by modulating the host innate immune system. These peptides have been shown to be protective against both Gram-positive and -negative pathogens but lack significant antimicrobial activity. In addition, these peptides selectively modulate pathogen-induced inflammatory responses and may be effective in countering dysregulation of immune response in dangerous conditions such as sepsis. Previously, peptides such as IDR-1 have been shown to act through signalling pathways including mitogen-activated protein kinase in monocytes and macrophages. Here we report a novel analysis of host gene expression response to synthetic peptide or the human cathelicidin (LL-37) peptide alone or in combination with bacterial endotoxin (lipopolysaccharide). A statistical analysis of the differential gene response in the context of the unified network of all known gene and protein interactions was performed, and identified a small module of twenty-six genes. This module appears to be central to host response to both LPS as well as peptides that counter the effects of LPS.

LB20

Aggregation of FcγRI Activates FcγRIIa Signaling in U937 Cells

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Aggregation of Fc_γ receptors ($Fc_\gamma Rs$) leads to a number of cellular responses, including the internalization of immune complexes, degranulation, activation of respiratory burst and secretion of cytokines. $Fc_\gamma Rs$ link the innate and the adaptive immunity and are thus absolutely crucial in immunological functions. In humans, $Fc_\gamma RI$ (CD64), $Fc_\gamma RIIa$ (CD32a), and $Fc_\gamma RIII$ (CD16) are activatory IgG-receptors. $Fc_\gamma RI$ (CD64), the high affinity receptor, is constitutively expressed on monocyte and

macrophage lineage cells. The low affinity receptor, $Fc_{\gamma}RIIa$, is the most widely distributed human $Fc_{\gamma}R$ and it is the only human $Fc_{\gamma}R$ having its own "Immunoreceptor Tyrosine-based Activation Motif" (ITAM). $Fc_{\gamma}RIIb$ is an inhibitory receptor having "Immunoreceptor Tyrosine-based Inhibition Motif" (ITIM) thereby modulating the immune responses. $Fc_{\gamma}RI$ and $Fc_{\gamma}RIIa$ are coupled to lipid metabolizing enzymes PLD and PLC_{γ} respectively. Lipids play an important role in $Fc_{\gamma}R$ signaling but are poorly studied. Our study employed LCMS to profile the lipid mediators in $Fc_{\gamma}R$ signaling. We used U937 cells expressing only $Fc_{\gamma}RI$ and $Fc_{\gamma}RIIa$ and U937 cells transfected with $Fc_{\gamma}RIIb$ (BI). $Fc_{\gamma}RI$ and $Fc_{\gamma}RIIa$ were aggregated using specific antibodies. MEK and ERK family of MAPKs were optimally activated both in U937 and BI cells at 10 min of aggregation indicating downstream $Fc_{\gamma}R$ signaling. The LCMS analysis of $Fc_{\gamma}RI$ aggregation revealed higher phosphatidic acid (PA) species in U937 cells indicating the activation of PLD. Interestingly, PA 38:4 species was approximately two fold higher in U937 cells than in BI cells. Polyunsaturated PA species are derived from PLC_{γ} and DGK catalyzed reactions. Our data therefore indicates that aggregation of $Fc_{\gamma}RI$ also activates $Fc_{\gamma}RIIa$ coupled PLC_{γ} signaling. This work was supported by Biomedical Research Council Grants R-183-000-211-305,Singapore

LB21

Decreased Expression of the LPS Receptor (CD14) on Human Monocytes Induced by Intravenous Immunoglobulins (IVIg)

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CD14 is a cell surface glycoprotein expressed mainly on monocytes/macrophages which recognizes lipopolysaccharide (LPS) and other pathogen-associated molecular patterns. CD14 can also be found in a soluble form (sCD14) in normal human plasma and was previously shown to be involved in the maintenance of lymphocyte homeostasis by acting as a negative regulator of T cell activation and function. Therapeutic preparations of plasma-derived human IgG (IVIg) also exhibit anti-inflammatory effects leading to a decreased T cell activation and IL-2 secretion. However, the mechanisms responsible for these anti-inflammatory effects are not fully understood. In the present work, we show that IVIg inhibited IL-2 secretion in a mixed lymphocyte reaction (MLR), an in vitro model of allograft rejection. Since we previously showed that IVIg did not act directly on human T cells, we hypothesized that monocytes were the target of IVIg and postulated that anti-inflammatory effect of IVIg could occur following the release of sCD14 from monocytes. To directly assess the effect of IVIg on CD14, monocytes were purified from PBMC and incubated for 24 hours in presence or absence of 5 mg/ml of IVIg. The cells were then recovered, washed, labelled with a mouse anti-human CD14 APC conjugate and analyzed by flow cytometry. The results obtained showed that about 50% of monocytes lost mCD14 expression in the IVIg-treated cultures, suggesting that IVIg treatment induced the release of mCD14. The reduction in mCD14 was not observed in presence of F(ab')2 fragments of IgG prepared by pepsin-digestion of IVIg, suggesting that the effect of IVIg is Fc-dependent. Interestingly, F(ab')2 fragments of IVIg were also inefficient in reducing IL-2 secretion in MLR. Experiments to directly measure the presence of sCD14 in MLR done in presence of IVIg are underway and will permit to better characterize the role of CD14 in the anti-inflammatory effects of IVIg.

LB22

Novel Leukocyte Signaling Implicating 14-3-3 Proteins in Regulation of NADPH Oxidase 2

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Here we present a novel signaling mechanism by which reactive oxygen species (ROS) generation by NADPH oxidase 2 (Nox2) in human neutorphils is controlled by 14-3-3 proteins. Human neutrophils express six of the mammalian 14-3-3 isoforms (beta β, gamma γ, zeta ζ, epsilon ε, eta η, tau τ/theta θ).

Late Breaking Abstracts

Each monomer contains a pocket that binds a variety of phosphoproteins which often contain a defined 14-3-3 binding motif. Phosphorylation of some of the regulatory subunits ($p47^{phox}$, $p67^{phox}$) of Nox2 suggested to us that these proteins might interact with 14-3-3 proteins. A specific mutation at K50 within the binding pocket of 14-3-3 isoforms has a dominant negative (DN) effect that inhibits binding of the WT to its client proteins due to the formation of a mutant-WT dimer. Co-expression of 14-3-3γ DN with Nox2 and its regulatory subunits in HEK 293 cells increased ROS production by a remarkable 4-5- fold compared to the WT under PMA-stimulated conditions. Knockdown of several of the endogenous 14-3-3 proteins in HEK 293 cells using 14-3-3 isoform-specific siRNA increased ROS production by 2-3 fold compared to a control siRNA. These results suggested that some 14-3-3 isoforms may function as negative regulators of Nox2. In human neutrophils, endogenous 14-3-3 gamma and zeta co-immunoprecipitated with endogenous $p47^{phox}$ under either PMA or fMLF stimulated conditions. Phosphorylation of $p47^{phox}$ by PKC was evident under stimulated conditions using an antibody specific for phospho-Ser in a PKC motif. Immunoprecipitation of endogenous $p67^{phox}$ from human neutrophils indicated that $p67^{phox}$ was also phosphorylated by PKC. However, 14-3-3 isoforms did not co-immunoprecipitate with $p67^{phox}$. These data suggest that some 14-3-3 isoforms interact directly with phosphorylated $p47^{phox}$ and act as dampeners of ROS generation by Nox2 perhaps by sequestering phosphorylated $p47^{phox}$ in the cytosol, thereby limiting translocation of $p47^{phox}$ to the membrane.

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LB23

Rab27a promotes Neutrophil Chemotaxis through Chemokine-stimulated Mobilisation of Granule Subsets

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Neutrophil exocytosis is a critical process that allows mobilisation of cargo and membrane proteins important for adhesion, phagocytosis and killing. Previous studies have implicated Rab27, a member of the RabGTPase family of endocytic/exocytic regulators in the regulated secretion of granule subsets in neutrophils under optimal secretory conditions. Using physiological stimuli, we investigated the role of Rab27 in neutrophil chemotaxis. We find bone-marrow derived mouse neutrophils deficient in Rab27a (*ash/ash)* but not related isoform Rab27b (*Rab27b* KO) exhibit impairment in chemotaxis in response to Leukotriene B4 (LTB4) and Chemokine (C-X-C motif) ligand 2 (CXCL2). We confirm that F-actin polymerisation and chemokine receptor expression remain unaffected in *ash/ash* neutrophils. We assessed the relative mobilisation of granule subsets in response to LTB4 and CXCL2 and find specific granules are preferentially mobilised at low chemokine concentrations. Minor mobilisations of azurophilic granules and lysosomes were also observed. Assessment of *ash/ash* neutrophils highlighted defects in specific granule mobilisation at low chemokine concentrations. Our data indicates that Rab27a deficiency inhibits chemotaxis of neutrophils by a chemokine-independent mechanism. We suggest that Rab27 dependent mobilisation of specific granules during chemotaxis may provide an important source of membrane to drive neutrophil movement and supply chemokine receptors critical for maintenance of chemotactic response.

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LB24

Resolvin E1 Regulates Interleukin-23, Interferon-g and Lipoxin A4 to Promote the Resolution of Allergic Airway Inflammation

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Epidemiological studies have indicated that diets rich in omega-3 fatty acids are less prone to asthma, however the underlying mechanisms behind this observation remain unclear. Resolvin-E1 (RvE1) is an anti-inflammatory/pro-resolution lipid mediator derived from the omega-3 fatty acid eicosapentaenoic acid (EPA). Here we determined the impact of RvE1 on allergic airway inflammation using a murine model of asthma. RvE1 significantly blocked the development of allergic airway inflammation by ~80% and promoted the resolution of allergic airway inflammation when administered at the peak of inflammation, decreasing the infiltrating leukocytes by ~50% and decreasing the resolution interval from ~5 days to ~2.5 days. RvE1 administered at the peak of inflammation, improved airway hyper-responsiveness, and decreased mucus production. RvE1 selectively regulated peptide and lipid mediators, most notably decreasing IL-23 and IL-17 and increasing IFN- γ and the anti-inflammatory lipid mediator lipoxin A4. RvE1 blocked LPS induced release of IL-23 and IL-6 from bone marrow derived dendritic cells *in vitro*.

These results indicate that RvE1 is a potent regulator of allergic airway responses that can inhibit cytokines involved in the pathobiology of asthma, and suggest novel, pro-resolving therapeutic approaches for asthma.

LB25

Plexin-B2 Negatively Regulates Macrophage Motility and Rac Activation

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Plexins are cell surface receptors widely studied in the nervous system, where they mediate migration and morphogenesis though the Rho family of small GTPases. More recently plexins have been implicated in immune processes including cell-cell interaction, activation, migration and cytokine production. Plexin-B2 facilitates ligand induced cell guidance and migration in the nervous system through Rho GTPases. Our group shows that Plexin-B2 is highly expressed on cells of the innate immune system, including macrophages, conventional dendritic cells, and plasmacytoid dendritic cell. However it does not have detectable effects on the production of proinflammatory cytokines, phagocytosis of a variety of targets, or directional migration towards chemoattractants. Instead, *Plxnb2*$^{-/-}$ macrophages have greater cellular motility than wild type, which is accompanied by more active, GTP-bound Rac. These data show that Plexin-B2 functions in macrophages as a negative regulator of RacGTPase and cell motility.

LB26

Therapeutic potential of cationic proteins in inflammatory disorders : an alternative to IVIg.

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The inflammatory response is initiated by the presentation of antigens by antigen presenting cells (APCs) to CD4+ T cells. Defects in this process may lead to loss of tolerance and contribute to the initiation of autoimmune or inflammatory disorders. Intravenous immunoglobulins (IVIg) are therapeutic preparations of IgG obtained following fractionation of thousands of liters of human plasma. IVIg are known to induce anti-inflammatory effects in an increasing number of diseases when infused at very high doses. Therefore, this therapeutic blood-derived product is exposed to considerable risks of shortage and efforts have been undertaken to better understand the mechanisms of IVIg immunomodulatory effects, in order to develop potential substitutes. We recently reported that

IVIg inhibited the MHC II-restricted antigen-specific $CD4^+$ T cell activation induced by the presentation of native antigens, therefore reducing the overall inflammatory response. This effect was linked to the spontaneous internalization of IVIg inside APCs. In the present work, we show that cationic IgG (pI>8,5) contained in IVIg preparations are more efficiently internalized by APCs and exhibit a higher specific inhibitory activity on antigen presentation. To confirm the importance of the high pI, IVIg and human serum albumin (HSA), another blood-derived protein which is normally not internalized inside APCs, were chemically cationized. Internalization of cationized IVIg (cIVIg) and HSA (cHSA) was monitored by intracellular flow cytometry. The effect of cIVIg and cHSA on antigen presentation by APCs was evaluated using ovalbumin-specific CD4+ T cells, as previously described. Our results show that cIVIg and cHSA were more efficiently internalized by APCs and were much more efficient to inhibit the MHC II-restricted ovalbumin presentation than their native counterpart. Altogether, these results suggest that cationic proteins may have superior anti-inflammatory effects compared to IVIg for the treatment of autoimmune or inflammatory disorders and may represent potential candidates for the preparation of IVIg substitutes.

LB27

Effect Of Palmitate Treatment On Macrophage-Macrophage And Macrophage-Neutrophil Crosstalk

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Background: Obesity is a worldwide epidemic with increasing rates in both adults and children. It has been recognized that obesity is associated with chronic low grade inflammation characterized by increased inflammatory cytokine production, which contribute to the advent of insulin resistance in many obese individuals. Macrophages that infiltrate adipose tissue in obesity and high fat feeding are recognised as a major source of these cytokines. In addition, it has been hypothesized that these macrophages secrete chemokines attracting other macrophages and immune cells, which will in turn secrete cytokines and chemokines leading to the vicious cycle of inflammation seen in obesity.

Objectives: To determine if the exposure of macrophages to an obesogenic environment lead to immune cell attraction, and to characterize the factors and pathways activated in macrophages in response to this environment.

Results: Treatment of primary rat macrophages with the saturated fatty acid palmitate, an abundant saturated fatty acid in the western diet and a known trigger of inflammation, led to production of factors that attracted both macrophages and neutrophils. In addition, we noted activation of inflammatory pathways (JNK, p38 MAPK) and increased gene expression of pro-inflammatory cytokines (IL-6, TNFα) and chemokines (KC, MCP-1). Characterization of the secretome of the palmitate treated macrophages revealed the presence of multiple cytokines and chemokines in the conditioned medium, of which some are strong candidates for the observed macrophage and neutrophil attraction.

Conclusion: Exposure of macrophages to palmitate resulted in production of pro-inflammatory cytokines and chemokines that attracted macrophages and neutrophils, and this occured via activation of inflammatory signaling pathways. We are currently determining how the potential recruitment of macrophages and neutrophils by palmitate-activated macrophages in vivo is reflected by their presence in metabolically active tissues.

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Lisbeth N. Fink is employed at the Hagedorn Research Institute, Novo Nordisk A/S, Gentofte, Denmark.

LB28

The Importance of Salmonella enterica serovar Typhimurium SPI2 Effectors During Murine Typhoid Fever and Macrophage Infection

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Salmonella enterica are Gram-negative bacterial pathogens responsible for typhoid fever and gastroenteritis. After ingestion, *Salmonella* cross the intestinal epithelial barrier through M-cells in Peyer's patches, followed by macrophage and dendritic cell uptake, enabling their spread to systemic sites during typhoid fever. *Salmonella* use two type three secretion systems encoded by *Salmonella* pathogenicity islands 1 and SPI2 to inject virulence proteins, termed effectors, into host cells to modify cellular functions. SPI1 is involved in host cell invasion; SPI2 is required for intracellular survival and replication within phagocytes. Because SPI2 is crucial for survival and replication in macrophages, the role of SPI2 effectors continues to be a subject of interest. This work examines the contribution of SPI2 effectors to bacterial virulence in an *in vivo* model of murine typhoid fever, and models of macrophage and epithelial cell infection. A PCR-based approach was used to make unmarked, in-frame deletions of SPI2 effectors in *S. enterica* serovar Typhimurium. We then studied the virulence of mutants lacking SPI2 effectors in the *in vivo* and cell culturemodels. For the typhoid model, bacterial strains were orally administered to C57BL/6 mice, which were sacrificed 5 days post-infection. Systemic and intestinal organs were collected and analyzed for colony forming units. We found that the Δ*spvB*, Δ*sseF,* and Δ*spiC* mutants are attenuated for colonization in the intestinal and systemic sites. In HeLa epithelial cells, all mutants replicated to the same extent as wild-type. In RAW264.7 macrophages, the *ΔspiC,* and *ΔsteA* strains replicated poorly in comparison to wild-type *Salmonella*. This work confirms the importance of SPI2 effectors in macrophage replication and systemic spread. This study is important for the comparison of the roles of SPI2 effectors in virulence in different models, and provides a useful, comprehensive tool for the study of the interactions between *Salmonella* and the host.

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LB29

Macrophages Release Novel tRNA Synthetase Domain as Physiological Modulator of TLR Pathways in the Mouse

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Understanding of Toll-like receptors (TLRs) in mammalian physiology continues to expand with respect to innate immunity/homeostatic mechanisms, foreign ligands, and endogenous coupling proteins. A particular challenge is the identification and characterization of endogenous protein ligands. Along with functional analysis in the mouse, methods of cell biology, biochemistry, bioinformatics and high-resolution protein crystallography were used to identify a released, extracellular fragment from macrophages-- a key cell component of TLR biology. This N-terminal fragment ($AspRS1^{N1}$) arises from a surprising parent – aspartyl-tRNA synthetase (AspRS1), a member of the cytosolic aminoacyl-tRNA synthetase family. Previously, cytosolic AspRS1's sole function was thought to catalyze protein synthesis. Unlike previous studies of endogenous mammalian ligands for TLRs, we identified a distinct eukaryote-specific helix that confers MyD88-dependent agonism specifically through TLR2/TLR4 pathways. During the evolution of prokaryotes and lower eukaryotes, the $AspRS1^{N1}$ – helix was appended to AspRS1 with positive charges (SKK) to strengthen tRNA binding, and then substituted with negative charges (EED) in higher eukaryotes to confer full agonist activity of $AspRS1^{N1}$ in the TLR2/TLR4 pathways. The results suggest evolutionary TLR pathway mechanisms, where a genetically stable, cytosolic tRNA synthetase gene is expropriated

during evolution to permit tailoring of an extracellular modulator for signaling in or between tissues.

LB30

Activation of TAK1 by chemotactic factors and GM-CSF, and its impact on human neutrophil signaling and function

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Neutrophils are a pivotal component of the innate immune system. These professional phagocytes generate a host of cytokines and chemokines that modulate the inflammatory response. Formylated peptides, (like fMLP) and growth factors (like GM-CSF) are present in many inflammatory loci where neutrophils predominate, and induce such responses as cytokine production and delayed apoptosis in neutrophils. The MAP3K, TAK1, is implicated in cytokine production and upstream signaling in response to LPS, IL-1 and TNF α in several cell types, including neutrophils. Here, we show that TAK1 also participates in intracellular signaling induced by fMLP and GM-CSF. We found that TAK1 is rapidly and transiently activated by these stimuli in neutrophils. Inhibition of TAK1 leads to decreased ERK1/2 phosphorylation, whereas that of other kinases such as p38 MAPK and Akt is unaffected. Accordingly, inhibition of TAK1 or of the MEK/ERK pathway decreases the fMLP- or GM-CSF-elicited gene expression and secretion of cytokines in neutrophils (IL-8, IL-1 β , IL-1ra). Finally, TAK1 inhibition can partially revert the antio-apoptotic action of GM-CSF or chemoattractants. Given its central role in neutrophil signaling in response to various classes of stimuli, TAK1 represents a potentially promising therapeutic target.

LB31

Cellular LPS Binding Protein (LBP) Inhibits Responses of Innate Immunity – A Naturally Occuring Homozygous Mutation Leads to Hyperinflammation

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Lipopolysaccharide Binding Protein (LBP) is an acute-phase protein recognizing bacterial ligands and inducing activation of the innate immune system. While in the past investigations have focused on the analysis of the soluble LBP, it recently has been proposed that it also can integrate into cellular membranes. By overexpressing LBP in epithelial HEK293 cells that are also overexpressing TLRs we show that cellular LBP inhibits the inflammatory response brought about by bacterial ligands. These results were confirmed by analyzing peritoneal macrophages from LBP k.o.- and wt mice: macrophages lacking cellular LBP reacted stronger to a bacterial stimulation with cytokine release. In addition we stimulated blood cells obtained from volunteers genotyped for a frequent single nucleotide polymorphism (SNP) within the LBP gene. Individuals carrying the mutation in its homozygous or heterozygous form were compared with wt individuals. This mutation has been shown by us previously to lead to a lack of function regarding the capacity of binding microbial cell wall compounds. In line with the experiments employing the HEK293 cells, cells obtained from individuals homozygous fort the mutation were hyperreactive to stimulation with bacterial ligands. In order to confirm that the mutation leads to a pro-inflammatory situation we also expressed the mutated LBP in HEK293 cells and compared their responsiveness to cells over expressing wt-LBP. These cells again were highly sensitive to stimulation by LPS and other bacterial ligands, comparable to cells not over expressing LBP. In summary we here show that cellular LBP has an important function in inhibiting the responsiveness to bacterial

ligands. A naturally occurring lack-of-function mutation may lead to a hyperinflammatory state, and we propose that this is caused by a lack of inhibition of the innate immune response. These findings may have implications for disease susceptibility of individuals carrying this mutation, and analyses to investigate this are currently underway.

LB32

The Endocannabinoid 2-Arachidonoyl-Glycerol Activates Human Neutrophils: Critical Role of Leukotriene B_4

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Although endocannabinoids are important players in nociception and obesity, their roles as immunomodulators remain elusive. The main endocannabinoids described so far, namely 2-arachidonoyl-glycerol (2-AG) and arachidonyl-ethanolamide (AEA), induce an intriguing profile of pro- and anti-inflammatory effects. This could relate to cell-specific cannabinoid receptors expression and/or the action of endocannabinoid-derived metabolites. Importantly, 2-AG and AEA comprise a molecule of arachidonic acid (AA) in their structure and are hydrolyzed rapidly. We postulated that 1) the released AA from endocannabinoid hydrolysis would be metabolized into eicosanoids; 2) these eicosanoids would mediate some of the effects of endocannabinoids. To confirm these hypotheses, experiments were performed in which freshly isolated human neutrophils were treated with endocannabinoids. Unlike AEA, 2-AG stimulated degranulation, kinase activation and calcium mobilization by neutrophils. Although 2-AG was not chemotactic, it induced the release of a chemotactic activity for neutrophils. 2-AG also rapidly (1 minute) induced a robust biosynthesis of leukotrienes, similar to that observed with AA. The effects of 2-AG were not mimicked nor prevented by cannabinoid receptor agonists or antagonists, respectively. Finally, the blockade of either 2-AG hydrolysis, LTB_4 biosynthesis or BLT_1 receptor activation prevented all the effects of 2-AG on neutrophil functions. In conclusion, we demonstrated that 2-AG potently activates human neutrophils. This is the consequence of 2-AG hydrolysis, *de novo* LTB_4 biosynthesis, and an autocrine activation loop involving BLT_1. This study suggests that endocannabinoids and their metabolites wilol very likely play an important role in the regulation of inflammation *in vivo.*

LB33

Automated lipid A structure assignment by a hierarchical tandem mass spectrometry algorithm

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Lipopolysaccharide (LPS) is the primary molecule present in the outer membrane of gram-negative bacteria. The components of LPS, O-antigen, a core-polysaccharide and lipid A, active activate the host innate immune system via Toll receptor proteins. Previous studies have shown that alteration of lipid A structure greatly affects bacterial pathogenicity. Such structural diversity is often found in both the position and composition of the fatty acids, degree of phosphorylation, and number/type of terminal monosaccharide modifications. While infusion-based electrospray ionization (ESI) coupled to multiple-stage tandem mass spectrometry (MS^n) has been used to demonstrate lipid A structural diversity (Shaffer et al. 2007. J Am Soc Mass Spectrom;18:1080-92), annotation of the spectra

has remained a manual, expert driven, and slow process. In order to annotate lipid A MS^n tandem mass spectra in a more automated fashion, we devised a computational method to interpret the data that we refer to as Hierarchical Tandem Mass Spectrometry (HiTMS) algorithm. As a first-pass tool, HiTMS aids interpretation of lipid A MS^n data by providing the analyst with a set of candidate structures that may then be manually confirmed/refuted at a more rapid rate than manual verification alone. HiTMS uses prior structural knowledge of given lipid A species to decipher MS^n spectra from species-specific signature ions (e.g. Y- and Z-type ions) and neutral losses. Results are objectively evaluated via a mathematical scoring routine that assigns a cross-correlation score between theoretical and acquired tandem mass spectra. At a false discovery rate < 0.01, HiTMS correctly assigned 85% of the structures in a library of 133 manually annotated *Francisella novicida* lipid A structures. Additionally, HiTMS correctly assigned 85% of the structures in smaller library of the chemically distinct lipid A species from *Yersinia pestis* suggesting it may be used across species regardless of the level of complexity.

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LB34

Synthetic cationic peptide IDR-1018 modulates human macrophage differentiation

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Macrophages are critical to innate and adaptive immune responses. They must develop and respond in a rapid and efficient manner to challenges in the micro-environment like an infection, driving responses towards classically activated (M1) or alternatively activated (M2) phenotypes. Natural host defense peptides (HDP) like LL-37 are known for their multiple immunodulatory activities such as chemoattraction and stimulation of cell differentiation. However, many natural HDP have certain issues including toxicity that reduce their potential use as pharmaceuticals. Therefore, synthetic peptides have been created to overcome these obstacles and enhance useful functions. Our lab has created a library of bactenecin derivative peptides that have substantially more potent ability to induce chemokines in human PBMCs and highly reduced or no toxicity. From this, Innate Defense Regulator (IDR)-1018 was selected. In this ongoing study, we tested the effect of IDR-1018 on macrophage differentiation, due to the importance of this cell type in protective immune responses by IDR peptides. Using transcriptional and protein analysis, we observed that IDR-1018 induces a unique signature of immune responses driving macrophage differentiation towards an intermediate M1-M2 state. Additionally, IDR-1018 was also able to reverse a pro-inflammatory (M1) phenotype. These data indicate that during macrophage differentiation, IDR-1018 would be enhancing anti-inflammatory functions while still maintaining certain inflammatory functions important for the resolution of the infection. In these times of multidrug resistant infections, novel pharmaceutical approaches like IDR-1018, which are based on modulating the immune system, could be an excellent approach to this problem.

LB35

Regulatory Molecules Involved in the Expression of T cell Immunoglobulin Mucin Domain-3 in Mast Cells and T Cells

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T cell immunoglobulin mucin domain-3 (TIM-3) is a regulator of T lymphocytes and mast cells.

Although altered TIM-3 expression has been revealed in some pathologic conditions, its transcriptional regulation has not been well explored. Here we analyzed the molecules controlling TIM-3 transcription and showed that up-regulation of TIM-3 transcription was inhibited by MEK inhibition in PMA-stimulated T cells as well as TGF-beta-treated mast cells. However p38 and JNK inhibitors did not affect TIM-3 transcription in both cells. While TGF-beta-induced TIM-3 transcription was inhibited by SMAD inhibitor in mast cells, the luciferase activity under TIM-3 promoter region (-1362kb ~ +144bp) did not show TGF-beta responsiveness. In contrast the luciferase activity under TIM-3 promoter region increased in PMA stimulated T cells. Our results suggest Erk and SMAD involvement in TIM-3 transcription in T cells and mast cells.

LB36

CD14 is essential for down-regulation of C5a receptor mRNA by E. coli and LPS.

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Background. The complement system plays an important role in innate immunity. Generation of the potent effector molecule C5a is important for recruitment of immune cells and for effective clearance of microbes. Uncontrolled C5a production during sepsis is associated with high mortality and immune dysfunction of granulocytes. Our aim was to study the regulation of the C5a receptors CD88 and C5L2 mRNA and surface expression on leukocytes in a human whole blood model of sepsis using inhibitors of essential upstream recognition molecules. Methods. Fresh human whole blood was incubated with whole Escherichia coli (E. coli) bacteria or LPS from E. coli for two hours in the presence of the C3 inhibitor compstatin, a C5aR antagonist or an anti-CD14 neutralizing monoclonal antibody (mAb). Target genes CD88 and C5L2 were measured by relative quantification using real-time PCR. Expression of cell surface receptors were measured by flow cytometry. Results: CD88 mRNA remained stable during the incubation period in the negative control whereas a marked spontaneous decrease was seen for C5L2. Both E. coli and LPS markedly (>50%) down regulated CD88 and C5L2 mRNA compared to the negative controls. This down-regulation was completely reverted by anti-CD14 mAb, whereas the complement inhibitors had no effect. These data were confirmed by using whole blood from a C5 deficient patient. Reconstitution with C5 had no effect on the E. coli-induced reduction of receptor mRNA expression, whereas anti-CD14 mAb fully restored the expression. Conclusion: For the first time CD14 is identified as a single key regulator of CD88 and C5L2 mRNA when challenged with E. coli and LPS, indicating a new point of cross-talk between complement activation and Toll-like receptors in Gram-negative sepsis.